



HAL
open science

Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*)

Ignacio Fernández, Francisco Hontoria, Juan Ortiz-Delgado, Yannis Kotzamanis, Alicia Estévez, Jose-Luis Zambonino-Infante, Enric Gisbert

► To cite this version:

Ignacio Fernández, Francisco Hontoria, Juan Ortiz-Delgado, Yannis Kotzamanis, Alicia Estévez, et al.. Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). *Aquaculture*, 2008, 283 (1-4), pp.102-115. 10.1016/j.aquaculture.2008.06.037 . hal-04478588

HAL Id: hal-04478588

<https://hal.univ-brest.fr/hal-04478588>

Submitted on 27 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*)

Ignacio Fernández^{a,*}, Francisco Hontoria^b, Juan B. Ortiz-Delgado^c, Yannis Kotzamanis^d, Alicia Estévez^a, Jose Luis Zambonino-Infante^e and Enric Gisbert^a

^a IRTA, Centre d'Aquicultura, Crta. de Poblenu km 5.5, 43450 Sant Carles de la Ràpita, Tarragona, Spain

^b Instituto de Acuicultura de Torre de la Sal (CSIC), 12595 Torre de la Sal, Castellón, Spain

^c Andalusian Institute of Marine Sciences (CSIC), Campus Universitario Rio San Pedro, Apdo. Oficial, 11510, Puerto Real, Cádiz, Spain

^d Hellenic Centre for Marine Research, Institute of Aquaculture, Agios Kosmas, Hellinikon 16777, Athens, Greece

^e INRA-Ifremer, UMR Nutrition Aquaculture et Génomique, Centre de Brest, BP 70, 29280 Plouzané, France

*: Corresponding author : Ignacio Fernández, Tel.: +34 977745427; fax: +34 977443138, email address : Ignacio.Fernandez@irta.cat

Abstract:

Several nutritional studies have found a direct effect of several vitamins in chondrogenic and osteogenic development during early life stages of marine fish species. In the present study, the effect of vitamin A (VA) in gilthead sea bream skeletogenesis was evaluated by means of four different dietary regimes (enriched rotifers) containing increasing levels of total VA (75, 109, 188 and 723 ng total VA mg⁻¹ DW). Dietary treatments were offered to larvae during the rotifer-feeding phase (4–20 days after hatching), while later all groups were fed with *Artemia nauplii* and weaned onto the same inert diet. Different dietary doses of VA affected gilthead sea bream larval growth, survival, performance (maturation of the digestive system) and quality (incidence of skeletal deformities). Higher levels of dietary VA than those included in the commercial emulsion for rotifer enrichment led to different levels and typologies of skeletal deformities, indicating that gilthead sea bream larvae were very sensitive to small increases in dietary VA. The degree of ossification was affected by the level of VA in enriched rotifers: the higher amount of VA in the diet, the higher number of skeletal pieces ossified ($R = 0.585$, $P = 0.04$). Dietary VA affected the normal process of bone formation and skeletogenesis, the skeletal structures mostly affected by high amounts of dietary VA were those from the cranial skeleton (splanchnocranium), vertebral centrums and caudal fin complex. The premaxilla, maxilla and dentary bones were the cranial structures affected by dietary VA levels, resulting in a large incidence of animals with compressed snout. Dietary VA also affected the normal development of the opercular complex, and a dose–response dependant effect was observed in relation to the incidence of specimens with incomplete operculum. Body shape was also affected by the level of dietary VA, increasing the incidence of specimens with lordosis, kyphosis and/or scoliosis with the dose of VA, being the prehaemal and caudal vertebrae the most affected regions of the vertebral column with this kind of abnormalities. The caudal fin complex was the most affected region of the skeleton affected by dietary treatments as seen by the high incidence of skeletal deformities in fish fed different doses of dietary VA. Deformities affected all skeletal elements composing the caudal fin, although the most affected ones were, in order of importance, the epurals, hypurals, parahypural, neural arch and uroneurals. Differences in sensitivity to dietary VA amongst caudal fin skeletal elements might be due to their differential ontogenetic development and differences in the exposure time to VA.

An excess of dietary VA also accelerated the intramembranous ossification process of vertebral centrums leading to one or two supranumerary vertebrae, and a high incidence of fused and compressed vertebral centrums. The sensibility of the developing skeletal structures to dietary VA levels should incline us to test lower doses of VA in live preys enrichments during early larval stages and higher doses afterwards.

Keywords: Gilthead sea bream; *Sparus aurata*; Larval quality; Vitamin A; Skeleton; Deformities

67 **1. Introduction**

68 Gilthead sea bream is one of the most important marine fish species farmed in the
69 Mediterranean region with a total production of 71,355 t (FAO, 2005). In this area, a high
70 competence between aquaculture companies and a reduction of the gilthead seabream market
71 price due to overproduction during recent years, have forced aquaculture industry to reduce
72 their production costs and improving their larval rearing efficiency. Skeletal deformities and
73 their incidence are one of the most important factors affecting fish farmer's production costs,
74 determining the external morphology, growth and fish survival rate (Matsusato, 1986; Divanach
75 et al., 1997; Koumoundouros et al., 2002). In the aquaculture industry, losses due to
76 deformities occur at two levels. At hatcheries, reducing larval survival rate and growth
77 efficiency in malformed fish; and at on-growing farms, where malformed market size fish have
78 to be discarded or sold at lower values than market prices. The levels of losses at either point
79 are different, depending on the species and the husbandry practices followed. In whatever
80 circumstance, these losses are substantial, in terms of productivity and profitability, since
81 skeletal deformities might affect up to the 30 % of the production. This fact represents one of
82 the bottlenecks in actual marine aquaculture. The yearly production of more than 500 million
83 has a survival of less than 15-20%. High mortalities during the first stages of development,
84 which are typical for marine aquaculture, are responsible for a loss of several millions of euros
85 (Subasinghe, 1997). Thus, reducing the incidence of larval deformities would reduce the
86 economic cost of production, both in the hatcheries and in the out-growing production sectors,
87 and improve the quality of the products.

88 Most of skeletal deformities appear during the larval and juvenile stages, where many
89 biological processes take place for organogenesis, morphogenesis and metamorphosis in a
90 very short time. The development of skeletal disorders is linked to a poorly understood
91 relationship between nutrition, environment and genetic factors. The larval stage is a very
92 sensitive period where the harmonious larval development depends on the physiological,
93 environmental, genetic, xenobiotic and nutritional factors (see review in Lall and Lewis, 2007).

94 Among them, larval nutrition at first feeding is one of the key parameters that affect
95 skeletogenesis during early development. In this sense, several studies have demonstrated
96 that nutrients are responsible for the appearance of skeletal deformities when their level and/or
97 form of supply in the diet are inappropriate or unbalanced (Cahu et al. 2003, Lall and Lewis,
98 2007). The solution of the problem is strongly related to the understanding of the species- and
99 stage-specific environmental preferences and nutritional requirements of the fish larvae, as well
100 as to the ontogeny of the skeletogenesis and anatomy of each deformity type.

101 The effects of nutrition on bone development and remodelling have been deeply studied
102 in terrestrial vertebrates, whereas this information in fish is fragmentary depending on the
103 nutrient considered (Cahu et al., 2003; Lall and Lewis, 2007). In this sense, recent advances in
104 the composition of starter diets for marine finfish larvae and enriching emulsions for live preys
105 have identified several nutrients, particularly minerals, vitamins and lipids that can be critical for
106 normal skeletogenesis. Vitamin A (VA), a morphogenetic nutrient, includes all compounds that
107 possess the same biological activity of retinol, playing a key role in morphogenesis, cellular
108 differentiation and proliferation processes. VA determines normal growth, body patterning,
109 nervous system development, and differentiation of pigment cells, limbs and skeleton along
110 vertebrate early development (Ross et al., 2000). The fish are not able to synthesize VA, thus
111 they have to take it from diet and any excess or deficiency of this nutrient in the diet resulted in
112 abnormal growth and development (Dedi et al., 1995; Tarui et al., 2006; Takeuchi et al., 1998;
113 Haga et al., 2002 a, b; Villeneuve et al., 2005). The impact of dietary VA on fish larvae
114 development will depend on both the dietary dose and the developmental status of the larvae
115 at first feeding. It is then necessary to fine-tune this particular relationship for each species.

116 The objective of the present study was to evaluate the effect on larval performance
117 (growth, survival and maturation of the digestive function) and quality (incidence and typology
118 of skeletal deformities) of graded levels of dietary VA on gilthead sea bream larval
119 development.

120

121 **2. Materials and methods**

122 *2.1 Larval rearing and diets*

123 Gilthead sea bream larvae were obtained from a Spanish private hatchery and shipped to the
124 IRTA facilities. After their acclimation, larvae were distributed (initial density: 100 larvae L⁻¹) in
125 24 cylindrical tanks (100 L) connected to a recirculation unit (Carbó et al., 2002). Water
126 conditions were as follows: 18-19°C, 35 ppt salinity, pH between 7,8-8,2, 20% daily water
127 exchange and with gently aeration and oxygenation (> 4 mg l⁻¹) Photoperiod was 12L:12D, and
128 light intensity of 500 lux at water surface.

129 Larvae were fed from day 4 post hatch (dph) to 20 dph enriched rotifers (*Brachionus*
130 *plicatilis*, lorica length: 178±30 µm length), whose density was progressively increased from 5
131 to 10 rotifers ml⁻¹. *Artemia* nauplii (EG, INVE, Belgium) were offered to larvae from 16 to 22
132 dph, in increasing density from 0.5 to 2 nauplii ml⁻¹, and 2 days enriched-metanauplii from 20 to
133 40 dph (1 to 5 metanauplii ml⁻¹). From 36 dph to the end of the experiment (60 dph), larvae
134 were progressively weaned onto dry feed, first with Proton 1/2 and 1/4 (INVE, Belgium) and
135 then with Gemma Micro (size range: 75 to 500 µm; Skretting, Spain).

136 The effect of VA in gilthead sea bream skeletogenesis was evaluated by means of four
137 different dietary regimes containing graded levels of VA using enriched rotifers. As rotifers and
138 *Artemia* nauplii accumulate VA in different patterns (Giménez et al., 2007), it was not possible
139 to maintain the same levels of VA during all the life prey-feeding period. Thus, we decided to
140 focus our study during only the rotifer-feeding phase. The graded VA levels on live prey were
141 obtained adding retinol palmitate (1,600,000 IU g⁻¹, Sigma-Aldrich, Spain) to a commercial
142 enriching emulsion, Easy Selco™ (ES, INVE, Belgium). Theoretically, experimental emulsions
143 contained 450, 900, 2,250 and 4,500 ng retinol equivalents mg⁻¹ of emulsion in wet weight
144 (Table 1). Dietary treatments were named as R450, R900, R2,250 and R4,500 according to the
145 theoretical level of retinol contained in the enriching emulsion (wet weight). For comparative

146 purposes, the emulsion containing 450 µg retinol equivalents g⁻¹ (R450) was considered as the
147 control group (ES without retinol palmitate addition).

148 Live prey (rotifers and *Artemia* nauplii) were enriched according to Gimenez et al. (2007),
149 rotifers were enriched during two hours with 0.15 g of each experimental emulsion per litre, and
150 *Artemia* metanauplii for 18 h with 0.6 g ES l⁻¹. After enrichment, rotifers and *Artemia* were
151 gently siphoned from enriching tanks, collected in a mesh, and washed in freshwater to reduce
152 the bacterial load and rests of the enrichment emulsions. Live preys were introduced into the
153 rearing tanks three times per day in order to assure an optimal live prey density in the water
154 column, and their appropriate nutritional value.

155 The effects of graded levels of VA on gilthead sea bream larval performance and quality
156 was evaluated by quintuplicate (three tanks were used for regular sampling and two for final
157 survival). Larvae were sampled at 18 and 60 dph, coinciding with the end of the rotifer-feeding
158 phase and the end of the weaning period, respectively. For sampling purposes, larvae were
159 sacrificed with an overdose of anaesthetic (Tricaine methanesulfonate, MS-222, Sigma).

160

161 2.2 Biochemical analysis

162 Retinoids on enriching emulsions and live prey were analyzed by HPLC using a modification of
163 the method proposed by Takeuchi et al. (1998). After sampling, live prey were washed with
164 distilled water to remove marine salts and bacterial load, and samples were frozen at -80°C
165 until posterior analysis. Lipids were extracted with a chloroform:methanol mixture (C:M, 2:1)
166 according to the Folch method (Folch et al., 1957), and stored in C:M:BHT (2:1:0.01) at 20 mg
167 l⁻¹ at -20 °C until their analysis. Then, samples were evaporated and redissolved on
168 methanol:acetone (1:1 v/v) prior to their HPLC analysis. The HPLC system (Thermo Separation
169 Products, San Jose, CA, USA) was equipped with a Lichrospher C-18 reverse phase column
170 (Merck, Darmstadt, Germany) and a UV-visible detector set at a wavelength of 325 nm. The
171 mobile phase was a mixture (85:15 v/v) of 98% methanol with 0.5% ammonium acetate, and
172 chloroform. The flow rate was 1.5 ml min⁻¹ and the elution time was 18 min. The concentration

173 of each retinoid was calculated from the calibration curves constructed with the peak area
174 ratios of their external standards and an internal standard of retinol acetate added to the
175 samples. All the reference retinoids were purchased to Sigma-Aldrich (Spain).

176

177 *2.3 Larval growth and survival rate*

178 Sampled larvae ($n = 15$) from each tank were washed with distilled water to avoid marine salts
179 and used for body size and dry weight determination. Larval standard length (Ls) was
180 measured with digital camera connected to a binocular microscope Nikon SMZ 800, AnalySIS
181 (Soft Imaging Systems, GmbH). Once larvae were measured in length, they were dried at 60°C
182 until their weight was constant. Samples were weighted with an analytic microbalance Sartorius
183 BP211D. At the end of the experiment, the total length of 150 fishes from each rearing tank
184 was measured to evaluate the effects of VA on size dispersion. Survival rate was calculated as
185 the percentage of final surviving fish in relation to the initial number at the beginning of the trial.

186

187 *2.4 Maturation of the digestive system*

188 The activity of two intestinal brush border enzymes (alkaline phosphatase and aminopeptidase
189 N) and two pancreatic enzymes (trypsin and amylase) was used to assess the degree of
190 development and maturation of the digestive system of larvae fed graded levels of VA. Enzyme
191 activity was measured at 18 and 60 dph ($n = 50$ and 10 larvae per tank, respectively).

192 Sampled fish were washed with distilled water to avoid marine salts and stored at -80°C
193 prior to enzyme activity analysis. The whole 18 dph larvae were homogenized for enzymatic
194 assays, since they were too small to dissect, while older fish were dissected to separate
195 pancreatic and intestinal segments as described by Cahu and Zambonino-Infante (1994).
196 Samples were homogenized (Ultra-Turrax T25 basic, IKA[®] - Werke) in five volumes (v/w) of
197 ice-cold Milli-Q water, centrifuged at 3,300 g for 3 min at 4°C and the supernatant removed for
198 enzyme quantification. For determination of intestinal enzymes, samples were homogenized in

199 cold Mannitol 50 mM, Tris-HCl 2 mM buffer, pH 7.0. Intestinal brush border membranes were
200 purified according to the method developed for intestinal scrapping (Crane et al., 1979).

201 Trypsin (E.C. 3.4.21.4) activity was assayed according to Holm et al. (1988), at 25°C
202 using BAPNA (N- α -benzoyl-DL-arginine *p*-nitroanilide) as substrate. Amylase (E.C. 3.2.1.1)
203 activity was measured according to Métais and Bieth (1968), using soluble starch (0.3%)
204 dissolved in Na₂HPO₄ buffer pH 7.4 as substrate.

205 Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37°C using 4-nitrophenyl
206 phosphate (PNPP) as substrate (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was
207 determined at 25°C according to Maroux et al. (1973), using sodium phosphate buffer 80 mM
208 (pH = 7.0) and L-leucine *p*-nitroanilide as substrate (in 0.1 mM DMSO). Enzymatic activities
209 were expressed as the specific activity, milli-units per milligram of protein (mU/mg protein), and
210 soluble protein of crude enzyme extracts was quantified by means of the Bradford's method
211 (Bradford, 1976) using bovine serum albumin as standard. All the assays were conducted in
212 triplicate.

213

214 *2.5 Skeletal deformities analysis, observations and measurements*

215 To identify and quantify the skeletal deformities on larvae from different dietary treatments, 50-
216 60 larvae per each tank were sampled at the end of the experiment, and fixed in formaldehyde
217 solution (10%) until their double staining. Then, animals were stained for bone and cartilage on
218 whole mounts using a modification of the method described by Klymkowsky and Hanken
219 (1991). In brief, specimens were rehydrated two times in distilled water during 5 minutes and
220 then placed in alcohol 95°. Specimens were stained with alcian blue solution with 80% alcohol
221 95° and 20% glacial acetic acid during 24 hours, rehydrated through a graded series of alcohol
222 (95%–25%) and macerated using a 1% aqueous solution of KOH with 3% hydrogen peroxide
223 (9:1 in volume) until skeletal elements were clearly visible. Then, specimens were placed
224 between 6 and 20 hours in an aqueous solution saturated in sodium borate containing 0.3-0.5
225 g trypsin, and stained with alizarin red S (stock solution: 1% alizarin red in 1% KOH) during 24

226 hours. Staining time was variable and depended on the size of the specimen. Finally, fish were
227 washed with distilled water, followed by a series of baths in 1% KOH to remove the excess of
228 dye in soft tissues, and placed through graded series of glycerine-KOH solutions.

229 After staining, fish were placed on their right side, in order to observe meristic
230 characters and skeletal abnormalities in the cranium, vertebral column and caudal fin complex.
231 Skeletal structures were identified and named according to Faustino and Power (1998, 1999,
232 2001). The study was focused on the mean number of vertebra and frequency of individuals
233 with abnormal number of vertebrae. Special emphasis was placed on the deformities occurring
234 in the cranial region (upper and lower jaws), vertebral column and caudal fin complex (hypurals
235 and parahypurals, epurals, uroneural, and specialized neural arch). In particular, we calculated
236 the frequency of individuals with lordosis, scoliosis or kyphosis, the total sum of deformities in
237 the vertebral column, and the incidence of vertebral compression and fusion.

238 In order to assess the degree of ossification of gilthead sea bream juveniles and
239 establish a potential relationship with the dietary regimen during the larval stages, the
240 percentage of juveniles in different stages of ossification was determined at the end of the
241 experiment. Those stages were defined according to the ossification of selected bony
242 structures that better describes the ossification process in this species (Faustino and Power,
243 1998). In brief, stage I corresponded to the early ossification of some vertebral centrums; at
244 stage II, all vertebral centrums were completely ossified; and at the stage III, dorsal fin rays
245 were completely ossified. At stage IV, caudal fin rays were ossified, and at stage V, the
246 hypurals and parahypural started to ossify. The stage VI was characterized by the complete
247 ossificatfon of most of the skeletal structures with the exception of the uroneural 2 and the
248 haemal spines 2 and 3, which completed their ossification at older ages (larger sizes).

249

250 *2.6 Statistical analysis*

251 Results are given as means and standard deviations. Data expressed as percentage (survival,
252 incidence of skeletal deformities) were previously $\arcsin(x^{1/2})$ -transformed. Results were

253 compared by means of One Way ANOVA (data normally distributed, Kolmogorov–Smirnov
254 test) and when significant differences were detected the Tukey multiple-comparison test was
255 used to detect differences among experimental groups (Zar, 1974). The test of Kolmogorov-
256 Smirnov was used to evaluate the distribution of fish size at the end of the study. Correlation
257 between different variables was evaluated with the Pearson Product Moment Correlation test.
258 In all statistical analyses, the level of significant difference was set at $P < 0.05$. All the statistical
259 analyses were conducted using SigmaStat 3.0 (SPSS, Richmond, USA).

260

261

262

263 **3. Results**

264 *3.1 Retinoid content in experimental emulsions and live prey*

265 Total lipid and retinoid content in emulsions and rotifers enriched with graded levels of retinol
266 palmitate are presented in Tables 1 and 2, respectively. No statistically significant differences
267 were detected in the total lipid content of experimental emulsions and enriched rotifers with
268 different levels of VA (ANOVA, $P > 0.05$). Total vitamin A content in emulsions and enriched
269 rotifers increased with increasing levels of retinyl palmitate incorporated into the emulsion
270 (ANOVA, $P < 0.05$). Analysis of retinoid content of enriched rotifers showed that the real
271 incorporated level of total VA was 75, 109, 188 and 723 ng mg⁻¹ DW for live prey enriched with
272 R450, R900, R2,250 and R4,500 experimental emulsions, respectively (Table 2). Retinyl
273 palmitate (VA ester) was the dominant form of retinoid detected in emulsions and enriched
274 rotifers, whereas retinol (VA alcohol) was also detected but at a minor concentration. Either
275 retinal (aldehyde form of vitamin A) was not detected in emulsions or rotifers enriched with
276 graded levels of vitamin A, whereas low levels of retinoic acid (0.8-1.1 ng mg⁻¹ DW) were only
277 detected in enriched rotifers, although they were not significantly different amongst rotifers
278 enriched with graded levels of VA (ANOVA, $P > 0.05$).

279

280 *3.2 Larval growth and survival*

281 Table 3 contains the results of growth in length and DW of gilthead seabream larvae fed
282 different levels of VA. At 18 dph, no statistically significant differences were observed in DW in
283 larvae fed graded levels of VA during the rotifer-feeding phase, while fish fed rotifers enriched
284 with the control emulsion showed the best growth in length (ANOVA, $P < 0.05$). At the end of
285 the trial, larvae fed R450 and R900 were larger in length and DW (ANOVA, $P < 0.05$). Larvae
286 fed rotifers enriched with higher doses of total VA (R2,250 and R4,500) weighted 12 and 21%
287 less than the other groups. Similarly, the length of those fish was 3 and 7% smaller.

288 The frequency distribution of final Ls classes in experimental groups fed rotifers
289 enriched with R450, R900 and R2,250 followed a normal distribution. The Kolmogorov-
290 Smirnov test, however, revealed statistically significant differences between the final
291 distribution of Ls of fish fed rotifers enriched with the highest dose of total VA (R4,500),
292 skewing the distribution towards sizes classes comprised between 15 and 17 mm ($P < 0.05$;
293 Fig. 1).

294 Dietary levels of VA significantly affected fish larval survival (Table 3; ANOVA, $P <$
295 0.05). Final survival ranged from 2.9 to 9.1% depending on the experimental group. The
296 highest survival results were obtained in larvae fed R450 and R900, while higher dietary levels
297 of VA (R2,250 and R4,500) significantly reduce their viability.

298

299

300 *3.3 Maturation of the digestive system*

301 At the end of the rotifer-feeding period, the different VA levels in enriched rotifers significantly
302 affected the trypsin activity (ANOVA, $P < 0.05$, Fig. 2a), while no significant differences were
303 detected in amylase secretion (ANOVA, $P > 0.05$, Fig. 2b). At 18 dph, the specific trypsin in
304 larvae fed R450 was 2 times higher than in larvae from the other dietary treatments. At the end
305 of weaning (60 dph), no statistical significant differences in specific trypsin and amylase

306 activities were detected among larvae from different experimental groups (ANOVA, $P > 0.05$,
307 Fig. 2c, d).

308 The specific activity of intestinal brush border enzymes was also affected by the dietary
309 content of VA in enriched rotifers, although the trend in the specific activity of both enzymes
310 was different depending on sampling date. At the rotifer-feeding phase, the highest alkaline
311 phosphatase specific activity was measured in larvae fed the highest dose of VA in enriched
312 rotifers (R4,500) (ANOVA, $P < 0.05$; Fig. 3a), while specific activity of aminopeptidase N in the
313 above-mentioned group was the lowest amongst all four tested experimental groups (ANOVA,
314 $P < 0.05$; Fig. 3b). At 60 dph, the alkaline phosphatase specific activity in fish fed R450 and
315 R900 was 80% higher than those fed higher levels of VA (ANOVA, $P < 0.05$; Fig. 3c).
316 Aminopeptidase N specific activities followed the same trend than alkaline phosphatase. The
317 highest activities were recorded in fish fed R450 and R900, which were 4 times higher than
318 those in larvae fed R2,250; while intermediate values were detected in fish fed rotifers
319 containing the highest dose of VA (Fig. 3d).

320

321

322 *3.4 Skeletal deformities: typology and frequencies*

323 The typology and incidence of skeletal deformities in gilthead sea bream juveniles fed different
324 levels of VA during the rotifer-feeding phase are shown in Figures 4-11.

325 The degree of ossification was affected by the level of dietary VA in enriched rotifers
326 (Figure 4): the higher amount of VA in the diet, the higher number of skeletal pieces ossified (R
327 = 0.585, $P = 0.04$). Fish fed the highest dose of VA showed the highest frequency of specimens
328 in most advanced stages of ossification (69.6% in stages V and VI) in contrast with those fed
329 the control diet, which only showed the 39.2% of specimens. However, the above-mentioned
330 data were not significantly different due to large variability in the ossification process between
331 replicates from the same dietary treatment.

332 The presence of cranial skeletal deformities in the jaw apparatus was strongly
333 correlated to the level of retinoids in enriched rotifers ($R = 0.789$, $P = 0.002$) (Fig. 6a). The
334 premaxilla, maxilla and dentary bones were the cranial structures more affected by different
335 levels of dietary VA, resulting in large number of specimens with compressed snout
336 (pugheadness, underdevelopment of the maxilla and premaxilla, Fig 5). The incidence of jaw
337 deformities in larvae fed the highest doses of VA (R2,250 and R4,500) were significantly higher
338 than those observed in juveniles fed lower levels of VA (ANOVA, $P < 0.05$; Fig. 6a). No
339 statistical significant differences were detected regarding the incidence of jaw deformities
340 between larvae fed R450 and R900 (ANOVA, $P > 0.05$). However, fish fed R900 showed a
341 higher incidence of deformities in both jaws in the same specimen, while this type of
342 deformation was not observed in the control group (Fig, 6b).

343 The incidence of opercular deformities was significantly correlated to the dose of VA in
344 enriched rotifers ($R = 0.843$, $P = 0,001$; Fig. 6c). While the control group had $16.5 \pm 1.64\%$ of
345 the individuals with an incomplete operculum, the incidence of this kind of deformity reached
346 $39.2 \pm 3.05\%$, in those fish fed R4,500 (ANOVA; $P < 0.05$). Significant statistical differences
347 were also detected in the incidence of deformed operculum depending on the fish side
348 considered, indicating that the expression of this type of deformity was side-dependent. In all
349 experimental groups, the frequency of abnormal operculum was higher in the left than in the
350 right side, independently of the level of dietary VA (t-test, $P < 0.05$; Fig. 6d). However, no
351 statistically significant differences were detected in the frequency of individuals with bilateral or
352 right-sided opercular complex deformities (ANOVA, $P > 0.05$).

353 The number of vertebrae most frequently observed in gilthead sea bream is twenty-four.
354 At the end of the experimental period, statistically significant differences in the mean number of
355 vertebrae were detected between larvae fed R900 and the rest of dietary treatments (ANOVA,
356 $P < 0.001$; Fig. 7). Fish from this experimental group showed a higher frequency of specimens
357 with twenty-five (69.1%) and twenty-six vertebrae (24.2%). Those supranumerary vertebrae

358 were observed in the caudal region, between the urostyle and vertebra number 23, and were
359 significantly correlated to vertebral fusion and compression disorders ($R = 0.959$, $P < 0.0001$).

360 The incidence of specimens with lordotic, kyphotic or scoliotic bends in their vertebral
361 column tended to increase with increasing levels of total VA in enriched rotifers, although this
362 trend was not significant ($R = 0.564$, $P = 0.056$) due to the large variability of replicates.
363 Similarly, no significant differences could be detected between experimental groups (ANOVA,
364 $P > 0.05$; Fig. 8a, 9).

365 The frequencies of deformities in vertebral centrum were significantly affected by the
366 level of VA in the diet (Fig. 8b). In all treatments, the compression of vertebral centrum was
367 more frequent than their fusion with the adjacent ones (Fig. 8c, d). In particular, larvae fed
368 R900 showed the highest incidence of compressed (80%) and fused (40%) vertebral bodies
369 than the rest of the experimental groups (ANOVA, $P < 0.05$). The incidence of both types of
370 deformities in vertebral centrum was 65 and 33% more frequent in the above-mentioned
371 group than in larvae fed rotifers enriched with the control emulsion. Regarding the haemal and
372 neural spines of vertebrae, in the haemal region of the vertebral column, haemal spines were
373 significantly affected by the level of VA in the diet, increasing the incidence of twisted haemal
374 spines with the dose of dietary VA (ANOVA, $P < 0.05$). In contrast, no differences were
375 detected in the incidence of deformities in the neural spine (ANOVA, $P > 0.05$), although high
376 variability between replicates and dietary groups was detected.

377 The incidence of skeletal deformities along the vertebral column in gilthead sea bream
378 juveniles is shown in Figure 10. Independently of the level of VA in the diet, the caudal region
379 was the area most affected by compression and fusion of the vertebral centrum; although the
380 group fed R900 showed the highest percentage of compressed and fused vertebrae in this
381 region; mostly affecting the urostyle (30%) and vertebra number 23 (90%). Vertebrae from the
382 cephalic and prehaemal regions were also affected by the level of VA in the diet, thus juveniles
383 fed rotifers containing high levels of VA during the rotifer-feeding phase showed a higher
384 incidence of skeletal deformities in these regions ($>10\%$; ANOVA, $P < 0.05$).

385 The effect of different levels of dietary VA on the skeletogenesis of the caudal fin
386 complex is presented in Figure 11. Only the 29% of gilthead sea bream juveniles from the
387 control group had at least one skeletal anomaly per fish in the caudal fin complex, while more
388 than 90% of specimens fed higher levels of dietary VA had one or more types of deformities
389 per specimen (Fig. 11a). The skeletal elements in the caudal fin complex most affected were
390 the specialized neural arch, epurals, hypurals and parahypurals, and the uroneural. In most
391 cases, deformities consisted in twisted or undeveloped skeletal elements and their fusion with
392 adjacent ones. In particular, dietary levels of VA significantly affected the frequency of
393 specimens with deformed specialized neural arch (ANOVA, $P < 0.05$; Fig. 11b). Juveniles fed
394 R900 showed the highest frequency (50%) of specimens with this bonny element deformed in
395 comparison to the other dietary treatments containing higher levels of VA, while none of the
396 fish from the control group was detected with this type of skeletal deformity. The frequency of
397 individuals with deformed epurals, hypurals and parahypurals was significantly higher in those
398 treatments fed higher doses VA than in the control group (ANOVA, $P < 0.05$; Fig. 11c, d). The
399 increase in VA in the diet increased up to four and eight times the incidence of juveniles with
400 deformed epurals, hypurals, and parahypurals, respectively. The incidence of deformities in the
401 uroneural was lower than in the other caudal fin complex bonny pieces, affecting less than 5%
402 of fish (Fig. 11e), although no statistically significant differences were detected in the frequency
403 of specimens with deformed uroneural between different treatments due to the large variability
404 observed amongst replicates and experimental groups (ANOVA, $P > 0.05$).

405 Other minor skeletal deformities were also detected in gilthead sea bream juveniles,
406 such as supranumerary predorsal fin rays (<30%), and dorsal and ventral fin ray fusion (<13%),
407 although no statistical significant differences between experimental groups were detected in
408 the frequency of specimens with such skeletal anomalies due to the large variability observed
409 between dietary treatments and replicates. Due to their minor effect on the external
410 appearance and fish quality, data on these deformities was deliberately not included.

411

412 **Discussion**

413

414 Generally, marine fish larvae hatch much earlier in their development than other vertebrates,
415 suggesting that the spatiotemporal sequences of the skeletal development in teleosts are quite
416 different from those of higher vertebrates (Haga et al., 2002a). In gilthead sea bream larvae,
417 these developmental processes still continue after hatching, and this particularity facilitates
418 studies of the effects of nutrition on morphogenesis. In particular, several authors have
419 described the morphogenesis and osteogenesis processes in gilthead sea bream (Faustino
420 and Power, 1998, 1999, 2001; Koumoundouros et al., 1997a, b; 2002), while others have
421 focused their objectives in describing and quantifying the typology and incidence of skeletal
422 deformities in this species under different rearing conditions (Koumoundouros et al., 1997b,
423 2002; Chatain, 1994a; Andrades et al., 1996; Boglione et al., 2001). However, none of these
424 studies have evaluated the effect of the diet on skeletogenesis and appearance of skeletal
425 deformities during early ontogeny. In this sense, it has been recently demonstrated that the
426 morphogenesis of marine fish larvae could be perturbed by inappropriate dietary levels of
427 different nutrients (Takeuchi et al., 1998; Haga et al., 2002a, b; Villeneuve et al., 2005a, b;
428 Hernandez et al., 2006; Tarui et al., 2006). Thus, in the present study, we aimed to evaluate
429 the effects of different dietary levels of VA on the incidence of skeletal deformities and larval
430 performance in gilthead sea bream fed rotifers enriched with graded levels of this
431 morphogenetic nutrient.

432 The feeding protocol used in the present study makes difficult to perform accurate
433 nutritional studies, because of the variability of the nutrient content in live prey (Giménez et al.,
434 2007). However, the use of a balanced compound diet for this kind of study, as it has been
435 previously used in European sea bass (Villeneuve et al., 2005a, 2006), was discarded, since a
436 compound microdiet is not completely developed for first feeding gilthead sea bream. Lipid
437 content in rotifers after enrichment with graded levels of total VA was similar in all treatments.
438 This result indicates that the differences observed in total VA content in the live prey were only

439 due to the levels of retinyl palmitate and retinol incorporated into the experimental emulsions
440 and cannot be related to the emulsion preparation and/or the enrichment conditions. Under the
441 present experimental conditions, total VA levels in rotifers increased proportionally to the
442 content of retinyl palmitate in the enriching emulsion. In agreement with Takeuchi et al. (1998)
443 and Giménez et al. (2007), the increase in retinol and retinoic acid, this last form of retinoid not
444 present in the enriching emulsion, indicated that rotifers were able to absorb, digest and
445 metabolize the retinyl palmitate contained in the enriching emulsion. Although retinoic acid is
446 the most active form of VA (Ross et al., 2000), the similar concentration of this retinoid in all
447 batches of enriched rotifers with experimental emulsions suggested that the observed effects of
448 VA on gilthead sea bream larval development were not due to its content in rotifers, rather than
449 the accumulation and transformation of different forms of VA in larvae.

450 At the end of the present experiment, , the different levels of dietary VA used during the
451 rotifer-feeding phase (4-18 dph) significantly affected gilthead sea bream larval growth and
452 survival, indicating that early larval nutrition exerted a strong effect on the further larval
453 performance. Larvae that showed the highest growth in length and dry weight, and survival
454 were those fed rotifers enriched with R450 and R900 emulsions, while higher dietary doses of
455 VA dramatically reduced larval performance. Similar results have been reported in other fish
456 species, such as Japanese flounder (Dedi et al. 1995; Takeuchi et al., 1995, 1998; Haga et al.
457 2003), European sea bass (Villeneuve et al., 2005a), red sea bream (Hernández et al., 2006)
458 and Atlantic salmon (Ørnsrud et al., 2002) where high dietary doses of VA led to a lower growth
459 and survival. However, the results from the above-mentioned studies are not directly
460 comparable due to different experimental dietary levels of VA, feeding protocols and diets (live
461 prey and inert diets). Nevertheless, survival and growth results observed in gilthead sea bream
462 larvae fed the control diet were similar to those obtained in commercial hatcheries (Tandler et
463 al., 1995; Başaran et al., 2004), ensuring that valid physiological and nutritional observations
464 could be drawn from this study.

465 Correct maturation of the larval digestive system allows larvae digesting and
466 assimilating the ingested diet, incorporating the needed amount of nutrient required for normal
467 growth and harmonious development. Pancreatic and brush border intestinal enzyme activities
468 have been widely used in nutritional studies as markers of larval fish development (Zambonino-
469 Infante and Cahu, 2001). In this study, low trypsin specific activity at 18 dph in gilthead sea
470 bream larvae fed high levels of VA, might be indicative of a delay in the maturational process of
471 the exocrine pancreas, as Villeneuve et al. (2005a) already reported in European sea bass fed
472 inert diets containing different levels of dietary VA. However, dietary effects of VA
473 administrated during the rotifer feeding phase (4-18 dph) were not evident after larval weaning
474 at 60 dph, which might indicate that larvae fed high levels of VA were able to recover the
475 normal digestive status regarding the pancreatic enzymes, once the excess of VA was
476 eliminated from their diet (*Artemia* feeding and inert diet phases). In contrast to the results
477 reported by Villeneuve et al. (2006) with European sea bass fed high levels of VA, the specific
478 activity of amylase in gilthead sea bream was not affected by the dietary levels of VA, although
479 numeric values tended to decrease with dietary doses of VA, but they were not significant due
480 to large variability between replicates and experimental treatments.

481 The specific activity of brush border intestinal enzymes was also affected by the dietary
482 dose of VA. Alkaline phosphatase is considered to serve as a marker for the maturation of the
483 brush border of enterocytes: the greater its activity, the better the level of intestinal maturation
484 (Zambonino-Infante and Cahu, 2001). However, high recorded activities of this brush border
485 enzyme at 18 dph in fish fed the highest dose of VA (R4500) in comparison to the rest of
486 dietary treatments might be attributed to an increase in cell proliferation (hyperplasia) induced
487 by dietary VA (Reifen et al., 1998; Uni et al., 2000), rather than a more advanced stage of
488 maturation of the intestinal mucosa, as aminopeptidase N specific activities indicated (three
489 times lower than in the other groups). At the end of the study, the low brush border enzyme
490 activity (alkaline phosphatase and aminopeptidase N) found in larvae from R2250 and R4500
491 groups, indicated dietary VA interfered with the normal development of the intestinal mucosa

492 and consequently, this might have impaired normal larval growth and further development. It
493 has been reported in different vertebrate species that VA influences enterocyte proliferation
494 and maturation, and decreases brush border enzyme-specific activity (Reifen et al., 1998; Uni
495 et al., 2000; Villeneuve et al., 2005a).

496 Dietary VA also affected the normal process of bone formation and skeletogenesis in
497 gilthead sea bream. The skeletal structures most affected by high levels of dietary VA were
498 those from the cranial skeleton (splanchnocranium), vertebral centrums and caudal fin
499 complex. Many authors have reported that the operculum complex, premaxilla, maxilla and
500 dentary bones were the cranial structures mostly affected by skeletal deformities (Barahona-
501 Fernandes, 1982; Chatain, 1994b; Andrades et al., 1996; Francescon et al., 1988; Boglione et
502 al., 2001; Faustino and Power, 2001; Villeneuve et al., 2005). In this study, the premaxilla,
503 maxilla and dentary bones were the cranial structures affected by dietary VA levels, resulting in
504 a large incidence of animals with compressed snout. This kind of deformity is quite common in
505 gilthead sea bream intensive larval rearing conditions (Andrades et al., 1996; Loy et al., 1999;
506 Boglione et al., 2001) and it has been also reported in other finfish species (Barahona-
507 Fernandes, 1982; Haga et al., 2003; Villeneuve et al., 2005a, 2006). The high incidence of this
508 kind of deformity under current experimental conditions might be linked to the ontogeny of the
509 splanchnocranium formation in gilthead sea bream, since the maxillar, premaxillar and dentary
510 are some of the first skeletal structures to appear and ossify due to their important functional
511 roles (Faustino and Power, 2001), although the frequency of the detection might be influenced
512 by the fatal nature of these kinds of deformities (Barahona-Fernandes, 1982).

513 Under intensive rearing conditions, opercular abnormalities in gilthead sea bream can
514 affect up to 80% of the population, seriously compromising both fish morphology and biological
515 performance (Koumoundouros et al., 1997b). Considering that defects in the opercular
516 complex are frequent and have been reported in many different fish species (Barahona-
517 Fernandes, 1982; Beraldo et al., 2003; Fraser and Nys, 2005), this structure seems to be
518 fundamentally fragile and easily alterable during early development stages (Beraldo et al.,

519 2003). In the present study, opercular abnormalities in gilthead sea bream fed the control diet
520 were 15.0% and close to values reported by Galeotti et al. (2000). However, the level of dietary
521 VA affected the incidence of abnormal opercula, since the incidence of reduced opercula
522 increased with the levels of VA in enriched rotifers. Earlier studies on opercular deformities in
523 gilthead sea bream concluded that unilateral deformation was side-independent and hence it
524 was the result of a fluctuating asymmetry model (Koumoundouros et al., 1997b; Galeotti et al.,
525 2000; Beraldo et al., 2003). Surprisingly, under the present experimental conditions a high
526 frequency of reduced opercula was detected in the left side of the head. These results are in
527 agreement with those reported by Verhaegen et al. (2007) and suggested a directional
528 asymmetry model. Fluctuating asymmetry is believed to be a consequence of environmental
529 factors that have an effect on developmental instability during the early life stages (Barahona-
530 Fernandes, 1982; Koumoundouros et al., 1997b), while directional asymmetry is believed to be
531 an inherited factor. As Verhaegen et al. (2007) reported, the existing literature on the genetic
532 influence on the asymmetric development of opercular deformities is contradictory depending
533 on the species and study. Although a basic assumption of asymmetry research is that left and
534 right side experience identical environmental factors, this may not be the case under the
535 present rearing conditions where the hydrodynamics of the experimental tanks with a central
536 outlet might have caused a different opercular development on both sides of the body.
537 However, the aetiology of this type of skeletal deformity remains unclear and further studies
538 have to be conducted to elucidate if the cause is due to environment, to genetics or both.

539 Under the present experimental conditions, the levels of dietary VA affected the normal
540 skeletogenesis process of the vertebral column and the number of vertebrae. Other authors
541 have previously reported that the number of vertebrae in fish can be influenced by factors other
542 than nutrition, such as triploidy in trout (Kacem et al., 2004), or temperature in halibut (Lewis et
543 al., 2004). In gilthead sea bream, the mean number of vertebrae is twenty-four, although there
544 is some discrepancy in the literature about the frequency of individuals with one vertebra more
545 or less. In this sense, Bogliione et al. (2001) found 75% of fish with twenty-three and twenty-five

546 vertebrae, while the prevalence of fish with twenty-five vertebrae was only 5% according to
547 Faustino and Power (2001). Under the present experimental conditions, larvae fed the control
548 diet showed most part of fish with twenty-four vertebrae (75%) and a low incidence of vertebral
549 deformities. Differences between these data might be due to other factors than nutritional
550 conditions, and could be related to different rearing conditions (e.g. extensive and intensive
551 rearing systems) and origin of larvae (e.g. egg quality and/or broodstock diet). However, the
552 level of dietary VA had a marked effect on the normal process of morphogenesis of the
553 vertebral column, since higher levels of VA than those from the control diet resulted in a higher
554 incidence of individuals with supranumerary vertebrae and a higher incidence of vertebral
555 deformities (compression and/or fusion of vertebral centrums). The morphogenetic effects of
556 VA on the normal development of the vertebral column have also been reported in Japanese
557 flounder (Haga et al., 2002a), European sea bass (Villeneuve et al., 2006) and red sea bream
558 Hernández et al. (2006). High levels of dietary VA in gilthead sea bream were responsible for a
559 higher incidence of specimens with a supranumerary vertebra in the caudal region of the
560 vertebral column, while in European sea bass larvae resulted in a loss of one vertebra. As
561 vertebrae from the caudal region are the last to ossify in gilthead sea bream (Faustino and
562 Power, 1998), an excess of dietary VA might have accelerated the normal differentiation
563 pattern of vertebral centrums and their osteogenesis, resulting in one or two supranumerary
564 vertebrae. Our hypothesis is supported by recent results (Mazurais et al., 2008) showing that
565 the dietary level of vitamins positively influence osteogenesis differentiation. These differences
566 in the effect of high levels of dietary VA in the number of vertebral centrums between both
567 species might be due to differences in the timing of notochord segmentation and vertebral
568 centrums formation, although further studies considering the expression pattern of genes
569 involved in larval morphogenesis and skeletogenesis are needed for comparing results from
570 both studies.

571 Body shape was also affected by the level of dietary VA, increasing the incidence of
572 specimens with lordosis, kyphosis and/or scoliosis with the dose of VA in the diet, being the

573 prehaemal and caudal vertebrae the most affected regions of the vertebral column with this
574 kind of abnormalities. These results were similar to those reported in European sea bass
575 larvae, where the authors found a statistically significant linear correlation between the dietary
576 level of VA and the frequency of animals with deformities in their vertebral column (Villeneuve
577 et al., 2005a). These kinds of deformities affecting the normal development of the vertebral
578 column have been described to affect larval performance and survival (Faustino and Power,
579 2001; Koumoundouros et al., 2002); since they have been reported in specimens presenting a
580 smaller body size in comparison to non-affected fish (Koumoundouros et al., 2002). This kind
581 of skeletal deformities can also be induced by unfavourable rearing conditions, such as tank
582 hydrodynamics (Divanach et al., 1997), water temperature (Sfakianakis et al., 2006) or non-
583 inflation of the swimbladder (Chatain, 1994a). However, the absence of lordotic or kyphotic fish
584 from the control group indicated that the experimental rearing conditions were optimal for the
585 proper development of the skeleton, and consequently, skeletal abnormalities might be
586 attributed to the effect of dietary VA. Our data indicate that these anomalies in body shape
587 resulted from deformities (compression and fusion) of one or two vertebral centrums, as it was
588 previously reported in Japanese flounder (Takashima, 1978; Dedi et al., 1995) and red sea
589 bream (Hernandez et al., 2006). Deformities in vertebral centrums in the haemal and caudal
590 region of the vertebral column have been found to be significantly correlated with the number of
591 vertebrae. The appearance of supernumerary vertebrae reduced the space for the normal
592 development of the other centrums, which ended compressing the vertebrae (also referred to
593 as platyspondyly). In this sense, Faustino and Power (2001) showed a clear relationship
594 between the incidence of lordotic gilthead sea bream and the frequency of specimens with
595 twenty-three vertebrae. The appearance of vertebral fusions might be attributed to a defect of
596 notochord segmentation and disruption of vertebral centrum differentiation, which might be a
597 result of a VA-induced accelerated skeletogenesis. In addition, the higher incidence of vertebral
598 deformities in the cephalic and prehaemal vertebrae (15-18%) in fish exposed to the highest
599 VA level (R4,500) in contrast to the other dietary regimes (<5%), showed that high doses of VA

600 accelerated the normal differentiation pattern of vertebral centrums of these regions that
601 appear chronologically earlier (Faustino and Power, 1998).

602 The caudal fin complex was the most affected region of the gilthead sea bream skeleton
603 affected by dietary treatments as seen by the high incidence of skeletal deformities in fish fed
604 different doses of dietary VA. Deformities affected all skeletal elements composing the caudal
605 fin, although the most affected were, in order of importance, the epurals, hypurals, parahypural,
606 neural arch and uroneurals. The skeletal elements that compose the caudal fin (urostyle and fin
607 elements) are formed either by endochondral or by intramembranous ossification (Gavaia et
608 al., 2002). The first group included the parahypural, hypurals, epurals, uroneurals and the
609 specialized neural arch; and the second group included the vertebra 23, the urostyle and the
610 caudal fin rays. The present results indicate that independently of the ossification process type
611 that takes place in the different skeletal elements that compose the caudal fin, it seems that the
612 effect of dietary VA is the same, and might be more related to the intense ossification process
613 induced by the VA that disrupted the normal and harmonious development of the above-
614 mentioned skeletal elements. Differences in sensitivity to dietary VA amongst caudal fin
615 skeletal elements might be due to their differential ontogenetic development, and to differences
616 in the exposure time to VA. Thus, the skeletal structures more sensitive to dietary VA were
617 those that differentiated earlier, such as epurals, hypurals and parahypurals, whose cartilages
618 appeared between 4.4 - 5.1 mm Ls and were not completely ossified until 14.7 – 16.0 mm Ls
619 (Faustino and Power 1998). In contrast, the uroneurals, which appeared at larger sizes (10.4 –
620 16.0 mm Ls, Faustino and Power 1998) when all the elements of the caudal complex were
621 already ossified, were less affected by dietary treatments (<5%) due to its late ossification
622 process (Koumoundouros et al. 1997a; Faustino and Power 1998).

623 Although dietary VA affected the incidence of skeletal deformities in the caudal fin
624 complex, these abnormalities were not lethal but seriously affected the external appearance
625 and quality of larvae and juveniles, confirming the data reported by Koumoundouros et al.
626 (1997a). These results are in agreement with those already published from wild-caught and

627 hatchery reared gilthead sea bream (Koumoundouros et al., 1997a; Boglione et al., 2001),
628 although differences in the typology and frequencies of different skeletal deformities exist
629 amongst studies. The incidence of skeletal deformities in the caudal complex was affected by
630 the levels of dietary VA, the higher the VA dose, the higher the incidence of deformed skeletal
631 elements. Similar results have been obtained in summer flounder (Martínez et al., 2007), and
632 Japanese flounder exposed to different levels and forms of this nutrient by means of balneation
633 (Haga et al., 2002a) and dose-response dietary trials (Dedi et al., 1998; Haga et al., 2002b). In
634 contrast to Haga et al. (2002a, b), we did not find any case of partial or complete absence of
635 the caudal fin when larvae were exposed to high levels of dietary VA. Such differences
636 between different studies might be due to different levels of retinoic acid to which larvae were
637 exposed, independently to the experimental approach used (balneation or dose-response trial).
638 According to the former authors, retinoic acid would inhibit cartilage differentiation before the
639 commencement of chondrocytes formation, causing the loss of the hypural, which would result
640 in the loss of the caudal fin.

641 In conclusion, different dietary doses of VA affected gilthead sea bream larval growth,
642 survival, performance (maturation of the digestive system) and quality (incidence of skeletal
643 deformities). Higher levels of dietary VA than those included in the commercial emulsion for
644 rotifer enrichment led to different levels and typologies of skeletal deformities, indicating that
645 gilthead sea bream larvae were very sensitive to dietary levels of VA (an increase of only 1.5
646 times of total VA in enriched rotifers, significantly increased the incidence of skeletal
647 deformities). Dietary VA affected the normal process of bone formation and skeletogenesis, the
648 skeletal structures mostly affected by high amounts of dietary VA were those from the cranial
649 skeleton (splanchnocranium), vertebral centrums and caudal fin complex. An excess of dietary
650 VA also accelerated the intramembranous ossification process of vertebral centrums leading to
651 a supranumerary vertebra, and a high incidence of fused and compressed vertebrae. Further
652 studies are needed to evaluate the effect of VA on larval quality and the molecular mechanisms
653 involved in skeletogenesis. Moreover, the sensibility of the developing skeletal structures to

654 dietary VA levels should incline us to test lower doses of VA in live preys enrichments during
655 early larval stages and higher doses afterwards.

656

657

658 **Acknowledgments**

659 Authors would like to express their gratitude to J. Canoura, M. Monllaó and N. Gras for their
660 excellent technical assistance with live prey rearing. This work was funded by the Ministry of
661 Education and Culture (MEC) of the Spanish Government (project AGL2005-02478). The
662 collaboration with Y.K. (HMCR, Greece) was established thanks to the HG2004-0018 grant
663 (MEC, Spain). I.F was supported by a predoctoral MEC fellowship and E.G. by the Programa
664 Ramón y Cajal (MEC, Spain).

665

666

667 **References**

668

669 Andrades, J.A., Becerra, J., Fernandez-Llebrez, P., 1996. Skeletal deformities in larval, juvenile
670 and adult stages of cultured gilthead sea bream (*Sparus aurata* L.). *Aquaculture* 141, 1 –
671 11.

672 Barahona-Fernandes, M.H., 1982. Body deformation in hatchery reared European sea bass
673 (*Dicentrarchus labrax* CL.). Types, prevalence and effect on fish survival. *J. Fish Biol.* 21,
674 239-249.

675 Başaran, F., Muhtaroglu, G., Ilgaz, S., Boyacioğlu, H., 2004. The Effect of Tank Volumes on
676 Survival of Gilthead Seabream (*Sparus aurata* L., 1758) from Hatching to the First
677 Grading in Intensive Culture Systems. *E. U. J. Fisher. Aquat. Sci.* 21, 69-72.

678 Beraldo, P., Pinoso, M., Tibaldi, E., Canavese, B., 2003. Abnormalities of the operculum in
679 gilthead sea bream (*Sparus aurata*): morphological description. *Aquaculture* 220, 89-99.

680 Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. Rapid coloric method for determination of
681 alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem. 164, 321-329.

682 Boglione, C., Gagliardi, F., Scardi, M., Cataudella, S., 2001. Skeletal descriptors and quality
683 assessment in larvae and post-larvae of wildcaught and hatchery-reared gilthead sea
684 bream (*Sparus aurata* L. 1758). Aquaculture 192, 1–22.

685 Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram
686 quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248-
687 254.

688 Cahu, C., Zambonino Infante, J.L., 1994. Early weaning of sea bass *Dicentrarchus labrax*
689 larvae with a compound diet: effect on digestive enzymes. Comp. Biochem. Physiol.
690 109A, 213-222.

691 Cahu, C., Zambonino Infante, J.L., Takeuchi, T., 2003. Nutritional components affecting
692 skeletal development in fish larvae. Aquaculture 227, 1- 4.

693 Carbó, R., Estévez, A., Furones, M.D., 2003. Intelligent and multifunctional recirculation
694 system. Its application in research at CA-IRTA. Spec. Publ.-EAS 32, 171-172.

695 Chatain, B., 1994a. Abnormal swimbladder development and lordosis in sea bass
696 (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*). Aquaculture 119, 371– 379.

697 Chatain, B., 1994b. Quality assessment of marine fish larvae and juveniles. In: Kestemont, P.,
698 Muir, J., Sevilla, F., Williot, P. (Eds.), Symposium on Measures for Success - Metrology
699 and Instrumentation in Aquaculture Management, 23-25 March 1994, Bordeaux, France,
700 Aquaculture, 181-183.

701 Crane, R.K., Boge, G., Rigal, A., 1979. Isolation of brush border membranes in vesicular form
702 from the intestinal spiral valve of the small dogfish *Scyliorhinus canicula*. Biochim.
703 Biophys. Acta 554, 264-267.

704 Dedi, J., Takeuchi, T., Seikai, T., Watanabe, T., 1995. Hypervitaminosis and safe levels of
705 vitamin A for larval flounder *Paralichthys olivaceus* fed *Artemia* nauplii. Aquaculture 133,
706 135-146.

707 Dedi, J., Takeuchi, T., Hosoya, K., Watanabe, T., Seikai, T., 1998. Effect of vitamin A levels in
708 *Artemia* nauplii on the caudal skeleton formation of Japanese flounder *Paralichthys*
709 *olivaceus*. Fish. Sci. 64, 344-345.

710 Divanach, P., Papandroulakis, N., Anastasiadis, P., Koumoundouros, G., Kentouri, M., 1997.
711 Effect of water currents during postlarval and nursery phase on the development of
712 skeletal deformities in sea bass (*Dicentrarchus labrax* L.) with functional swimbladder.
713 Aquaculture 156, 145–155.

714 FAO Fishery and Aquaculture Information and Statistics Service. Aquaculture production 2005.
715 FAO yearbook. Fishery statistics. Aquaculture production. Vol. 100/2. Rome, FAO. 2007.
716 202p.

717 Faustino, M., Power, D.M., 1998. Development of osteological structures in the sea bream:
718 vertebral column and caudal fin complex. J. Fish Biol. 52, 11–22.

719 Faustino, M., Power, D.M., 1999. Development of the pectoral, pelvic, dorsal and anal fins in
720 cultured sea bream. J. Fish Biol. 54, 1094–1110.

721 Faustino, M., Power, M.D., 2001. Osteologic development of the viscerocranial skeleton in sea
722 bream: alternative ossification strategies in teleost fish. J. Fish Biol. 58, 537–572.

723 Folch, J., Lees, M., Stanley G.H.S., 1957. A simple method for the isolation and purification of
724 total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

725 Francescon, A., Freddi, A., Barbaro, A., Giavenni, R., 1988. Daurade *Sparus aurata* L.
726 reproduite artificiellement et daurade sauvage. Experiences paralleles en diverses
727 conditions d'elevage. Aquaculture 72, 273-285.

728 Fraser, M.R., Nys, R., 2005. The morphology and occurrence of jaw and operculum deformities
729 in cultured barramundi (*Lates calcarifer*) larvae. Aquaculture 250, 496-503.

730 Galeotti, M., Beraldo, P., De Dominis, S., D'Angelo, L., Ballestrazzi, R., Musetti, R., Pizzolito,
731 S., Pinosa, M., 2000. A preliminary histological and ultrastructural study of opercular
732 anomalies in gilthead sea bream larvae (*Sparus aurata*). Fish Physiol. Biochem. 22, 151–
733 157.

- 734 Gavaia, P.J., Dinis, M.T., Cancela, M.L., 2002. Osteological development and abnormalities of
735 the vertebral column and caudal skeleton in larval and juvenile stages of hatchery-reared
736 Senegal sole (*Solea senegalensis*). *Aquaculture* 211, 305–323.
- 737 Gimenez, G., Kotzamanis, Y., Hontoria, F., Estévez, A., Gisbert, E., 2007. Modelling retinoid
738 content in live prey: A tool for evaluating the nutritional requirements and development
739 studies in fish larvae. *Aquaculture* 267, 76-82.
- 740 Haga, Y., Suzuki, T., Takeuchi, T., 2002a. Retinoic acid isomers produce malformations in
741 postembryonic development of the Japanese flounder, *Paralichthys olivaceus*. *Zool. Sci.* 19,
742 1105–1112.
- 743 Haga, Y., Takeuchi, T., Seikai, T., 2002b. Influence of all-trans retinoic acid on pigmentation
744 and skeletal formation in larval Japanese flounder. *Fisheries Sci.* 68, 560–570.
- 745 Haga, Y., Suzuki, T., Kagechika, H., Takeuchi, T., 2003. A retinoic acid receptor-selective
746 agonist causes jaw deformity in the Japanese flounder, *Paralichthys olivaceus*. *Aquaculture*
747 221, 381– 392.
- 748 Hernandez-H, L.H., Teshima, S., Koshio, S., Ishikawa, M., Gallardo-Cigarroa, F.J., Alam, M.S.,
749 Uyan, O., 2006. Effects of vitamin A palmitate, beta-carotene and retinoic acid on the growth
750 and incidence of deformities in larvae of red sea bream *Chrysophrys major*. *Cienc. Mar.* 32,
751 195-204.
- 752 Holm, H., Hanssen, L.E., Krogdahl, A., Florholmen, J., 1988. High and low inhibitor soybean
753 meals affect human duodenal proteinase activity differently: in vivo comparison with bovine
754 serum albumin. *J. Nutr.* 118, 515–520.
- 755 Kacem, A., Meunier, F.J., Aubin, J., Haffray, P., 2004. Caractérisation histo-morphologique des
756 malformations du squelette vertébral chez la truite arc-en-ciel (*Oncorhynchus mykiss*) après
757 différents traitements de triploidisation. *Cybium* 28, 15–23.
- 758 Klymkowsky, M.W., Hanken, J. 1991. Whole-mount staining of xenopus and other vertebrates.
759 *Method. Cell Biol.* 36, 419-425.

760 Koumoundouros, G., Gagliardi, F., Divanach, P., Boglione, C., Cataudella, S., Kentouri, M.,
761 1997a. Normal and abnormal osteological development of caudal fin in *Sparus aurata* L. fry.
762 Aquaculture 149, 215–226.

763 Koumoundouros, G., Oran, G., Divanach, P., Stefanakis, S., Kentouri, M., 1997b. The
764 opercular complex deformity in intensive gilthead sea bream (*Sparus aurata* L.) larviculture.
765 Moment of apparition and description. Aquaculture 156, 165– 177.

766 Koumoundouros, G., Maingot, E., Divanach, P., Kentouri, M., 2002. Kyphosis in reared sea
767 bass (*Dicentrarchus labrax* L.): ontogeny and effects on mortality. Aquaculture 209, 49-58.

768 Lall, S.P., Lewis-McCrea, L., 2007. Role of nutrients in skeletal metabolism and pathology in
769 fish, an overview. Aquaculture 267, 3-19.

770 Lewis, L.M., Lall S.P., Eckhard Witten, P., 2004. Morphological descriptions of the early stages
771 of spine and vertebral development in hatchery-reared larval and juvenile Atlantic halibut
772 (*Hippoglossus hippoglossus*). Aquaculture 241, 47–59.

773 Loy, A., Boglione, C., Cataudella, S., 1998. Geometric morphometrics and morpho-anatomy: a
774 combined tool in the study of sea bream (*Sparus. Aurata*) shape. J. Appl. Ichthyol. 14, 104–
775 110.

776 Maroux, S., Louvard, D., Baratti, J., 1973. The aminopeptidase from hog-intestinal brush
777 border. Biochim. Biophys. Acta 321, 282-295.

778 Martinez, G.M., Baron, MP., Bolker, J.A., 2007. Skeletal and pigmentation defects following
779 retinoic acid exposure in larval summer flounder, *Paralichthys dentatus*. J. World Aquac.
780 Soc. 38, 353-366.

781 Matsusato, T., 1986. Study on skeletal anomaly of fishes. Bull. Natl. Res. I. Aquac. 10, 57-179
782 (in Japanese with English abstract).

783 Mazurais, D., Darias, M.J., Gouillou-Coustans, M.F., Le Gall, M.M., Huelvan, C., Desbruyères,
784 E., Quazuguel, P., Cahu, C., Zambonino-Infante, J.L., 2008. Dietary vitamin mix levels
785 influence the ossification process in European sea bass (*Dicentrarchus labrax*) larvae. Am.
786 J. Physiol. Regul. Integr. Comp. Physiol. 294, 520-527.

787 Métais, P., Bieth, J., 1968. Détermination de l'a-amylase par une microtechnique. Ann. Biol.
788 Clin. 26, 133-142.

789 Ornsrud, R., Graff, L.E., Hoie, S., Totland, G.K., Hemre, G.I., 2002. Hypervitaminosis A in first-
790 feeding fry of the Atlantic salmon (*Salmo salar* L.). Aquacult. Nutr. 8, 7-13

791 Reifen, R., Zaiger, G., Uni, Z., 1998. Effect of vitamin A on small intestinal brush border
792 enzymes in rat. Int. J. Vit. Nutr. Res. 68, 281-286.

793 Ross, S.A., Caffery, P.J., Dragner, U.C., De Luca, L.M., 2000. Retinoids in embryonal
794 development. Physiol. Rev. 80, 1021–1054.

795 Sfakianakis, D.G., Georgakopoulou, E., Papadakis, I.E., Divanach, P., Kentouri, M.,
796 Koumoundouros, G., 2006. Environmental determinants of haemal lordosis in European sea
797 bass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquaculture 254, 54-64.

798 Subasinghe, R., 1997. Fish health in quarantine. Review of the state of the world aquaculture.
799 FAO Fisheries Circular N° 886. Food and Agriculture Organisation of the United Nations,
800 Rome, 45-49.

801 Takashima, F., 1978. Vertebral malformation in hatchery-reared red sea-bream, *Chrysophrys*
802 *major*. Bull. Jpn. Soc. Sci. Fish. 44, 435-443.

803 Takeuchi, T., Dedi, J., Ebisawa, C., Watanabe, T., Seikai, T., Hosoya, K., Nakazoe, J.I., 1995.
804 The effect of Beta-carotene and Vitamin-A enriched *Artemia* nauplii on the malformation and
805 color abnormality of larval Japanese flounder. Fish. Sci. 61, 141-148.

806 Takeuchi, T., Dedi, J., Haga, Y., Seikai, T., Watanabe, T., 1998. Effect of vitamin A compounds
807 on bone deformity in larval Japanese flounder (*Paralichthys olivaceus*). Aquaculture 169,
808 155– 165.

809 Takeuchi, T., Dedi, J., Haga, Y., Seikai, T., Watanabe, T., 1998. Effect of vitamin A compounds
810 on bone deformity in larval Japanese flounder *Paralichthys olivaceus*. Aquaculture 169,
811 155–165.

812 Tandler, A., Anav, F.A., Choshniak, I., 1995. The effect of salinity on growth rate, survival and
813 swimbladder inflation in gilthead seabream, *Sparus aurata*, larvae. *Aquaculture* 135, 343-
814 353.

815 Tarui, F., Haga, Y., Ohta, K., Shima, Y., Takeuchi, T., 2006. Effect of *Artemia* nauplii enriched
816 with vitamin A palmitate on hypermelanosis on the blind side in juvenile Japanese flounder
817 *Paralichthys olivaceus*. *Fish. Sci.* 72, 256-262.

818 Uni, Z., Zaiger, G., Gal-Garber, O., Pines, M., Rozenboim, I., Reifen, R., 2000. Vitamin A
819 deficiency interferes with proliferation and maturation of cells in the chicken small intestine.
820 *Br. Poult. Sci.* 41, 410– 415.

821 Verhaegen, Y., Adriaens, D., De Wolf, T., Dhert, P., Sorgeloos, P., 2007. Deformities in larval
822 gilthead sea bream (*Sparus aurata*): A qualitative and quantitative analysis using geometric
823 morphometrics. *Aquaculture* 268, 156–168.

824 Villeneuve, L., Gisbert, E., Le Delliou, H., Cahu, C.L., Zambonino-Infante, J.L., 2005a. Dietary
825 levels of all-trans retinol affect retinoid nuclear receptor expression and skeletal
826 development in European sea bass larvae. *Br. J. Nutr.* 93, 1–12.

827 Villeneuve, L., Gisbert, E., Zambonino-Infante, J.L., Quazuguel, P., Cahu, C.L., 2005b. Effects
828 of lipids on European sea bass morphogenesis: implication of retinoid receptors. *Br. J. Nutr.*
829 94, 877–884.

830 Villeneuve, L., Gisbert, E., Moriceau, J., Cahu, C.L., Zambonino Infante J.L., 2006. Intake of
831 high levels of vitamin A and polyunsaturated fatty acids during different developmental
832 periods modifies the expression of morphogenesis genes in European sea bass
833 (*Dicentrarchus labrax*). *Br. J. Nutr.* 95, 677-687.

834 Zambonino-Infante, J.L., Cahu, C.L., 2001. Ontogeny of the gastrointestinal tract of marine fish
835 larvae. *Comp. Biochem. Physiol.* 130, 477– 487.

836 Zar, J.H., 1974. *Biostatistical Analysis*. Prentice Hall, Englewood Cliffs, NJ. USA.
837
838

839

840

841 **Figure captions**

842 Figure 1. Final size distribution of gilthead sea bream larvae fed rotifers enriched with graded
843 levels of vitamin A.

844

845 Figure 2. Specific enzyme activity in larvae 18 dph fed with the different diets for trypsin (a) and
846 amylase (b) expressed in U mg⁻¹ prot DW, and in larvae at 60 dph for trypsin in mU mg⁻¹ prot
847 DW (c) and for amylase in U mg⁻¹ prot DW (d). Different indexed letters show significant
848 differences between treatments (ANOVA, $P < 0.05$).

849

850 Figure 3. Specific enzyme activity in 18 dph larvae fed with the different diets measured in U
851 mg⁻¹ prot DW for alkaline phosphatase (a) and aminopeptidase N (b), and in larvae at 60 dph
852 (c) and (d), respectively. Different indexed letters show significant differences between
853 treatments (ANOVA, $P < 0.05$).

854

855 Figure 4. Degree of ossification (Stages I-VI) of larvae fed different levels of VA-enriched
856 rotifers. The description of each stage of ossification is presented in the text.

857

858 Figure 5. Different types of mandibular deformities. Fish with normal developed upper and
859 lower jaws (a), fish with lower jaw (dentary) deformed (b), fish with the upper jaw (premaxillar)
860 deformed (c). Abbreviations: An, angular; De, dentary; Fr, frontal; Mx, maxillar; Nas, nasal; Pm,
861 premaxillar; Ps, parasphenoid; Sc, sclerotic.

862

863 Figure 6. Cranial deformities in gilthead sea bream fed different levels of VA. Frequencies of
864 fish with mandibular (a, b) and opercular complex (c, d) skeletal deformities. Different letters
865 show significant differences between treatments (ANOVA, $P < 0.05$).

866

867 Figure 7. Frequency of gilthead sea bream larvae fed different doses of dietary VA with
868 different number of vertebrae.

869

870 Figure 8. Frequency of fishes fed different levels of VA with lordotic, kyphotic or scoliotic
871 vertebral column (a), with at least one vertebral deformity (b), fused vertebral centrums (c) and
872 compressed vertebral centrums (d). Different letters show significant differences between
873 treatments (ANOVA, $P < 0.05$).

874

875 Figure 9. Fish with severe lordosis and kyphosis on their vertebral column.

876

877 Figure 10. Incidence of vertebral deformities (vertebral compression and fusion) along the
878 vertebral axis in larvae fed rotifers enriched with R450 (a), R900 (b), R2,250 (c) and R4,500 (d)
879 emulsions.

880

881 Figure 11. Incidence of deformities in the caudal fin complex in gilthead sea bream fed graded
882 levels of VA. Percentage of specimens with at least one deformity in the caudal fin (a),
883 specialized neural arch (b), epurals (c), hypurals and parahypural (d) and uroneural (e).
884 Different indexed letters show significant differences between treatments (ANOVA, $P < 0.05$).

885

886 Figure 12. Different typologies of skeletal deformities in the caudal region of gilthead sea
887 bream fed different levels of VA. Normal caudal fin complex developed (a), caudal fin complex
888 with vertebral centra compressed (b), caudal fin complex with fused preural centra 2 and 3 (c),
889 caudal fin complex with fused hypurals and straight urostyle (d) and caudal fin complex
890 severe deformed with several vertebral centrum deformities (e). Abbreviations: Ep, epural;
891 Haem, haemal spine; Hyp, hypural; Na, specialized neural arch; Neur, neural spine; Un,
892 uroneural; Phyp, parhypurapophyses; PU₂, PU₃, preural centra 2 and 3; Vert, vertebral
893 centrum.

894

895

Table 1. Total lipid and retinoid content (retinyl palmitate, retinol and total VA) in experimental live prey enriching emulsions. Total lipid content is expressed as % DW and retinoid content is expressed as ng mg⁻¹ DW. Different letters within the same column show statistical significant differences between emulsions (ANOVA, $P < 0.05$).

Emulsion	Total lipids	Retinyl palmitate	Retinol	Total VA
R450	86.8±11.9	1690.9±295.91 ^d	6.6±1.05 ^b	1698.3±297.13 ^d
R900	94.8±5.73	3219.6±386.14 ^c	5.6±1.48 ^b	3226.3±383.95 ^c
R2,250	95.5±1.92	7973.2±768.84 ^b	6.8±0.25 ^b	7980.8±768.05 ^b
R4,500	96.2±2.90	16931.6±44.09 ^a	11.4±1.23 ^a	16946.7±443.18 ^a

Table 2. Total lipid and retinoid content (retinyl palmitate, retinol, retinoic acid and total VA) in rotifers enriched graded levels of retinol palmitate. Total lipid content is expressed as % DW and retinoid content is expressed as ng mg⁻¹ DW. Different letters within the same column show statistical significant differences between dietary groups (ANOVA, $P < 0.05$).

Emulsion	Total lipids	Retinyl palmitate	Retinol	Retinoic acid	Total VA
R450	8.3±0.78	66.7±12.20 ^c	7.6±0.84 ^c	1.0±0.17	75.4±38.72 ^c
R900	7.8±0.66	100.3±17.21 ^{b,c}	8.2±0.60 ^c	0.8±0.29	109.2±18.10 ^c
R2,250	7.1±0.59	139.1±0.90 ^b	49.8±7.72 ^b	1.1±0.28	187.6±9.95 ^b
R4,500	8.7±1.43	681.9±65.54 ^a	68.1±7.72 ^a	0.9±0.28	723.3±26.21 ^a

Table 3. Larval size in standard length (Ls) and dry weight (DW), and survival rate of gilthead seabream larvae fed different levels of vitamin A. Values are mean \pm standard deviation. Different letters within the same column show statistical significant differences.

	Ls (mm)		DW (mg)		Survival (%)
	18dph	60dph	18dph	60dph	
R450	5.33 \pm 0.48 ^a	16.90 \pm 2.82 ^a	0.087 \pm 0.028 ^a	74.61 \pm 8.84 ^a	9.1 \pm 1.9 ^a
R900	4.94 \pm 0.06 ^{bc}	17.23 \pm 2.36 ^a	0.094 \pm 0.017 ^a	69.31 \pm 10.24 ^{ab}	8.0 \pm 0.2 ^a
R2,250	4.66 \pm 0.75 ^c	16.40 \pm 2.48 ^{ab}	0.097 \pm 0.043 ^a	65.61 \pm 8.18 ^b	2.9 \pm 0.3 ^b
R4,500	5.11 \pm 0.84 ^{ab}	15.66 \pm 2.03 ^b	0.115 \pm 0.027 ^a	56.86 \pm 11.27 ^c	3.3 \pm 0.5 ^b

Figure 1
[Click here to download high resolution image](#)

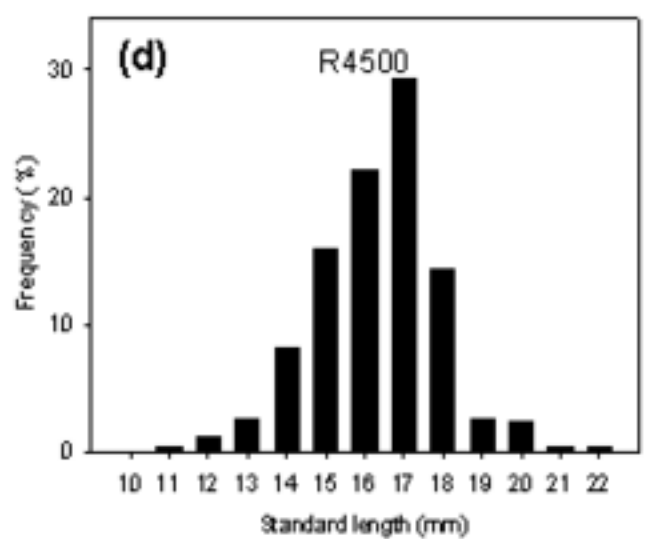
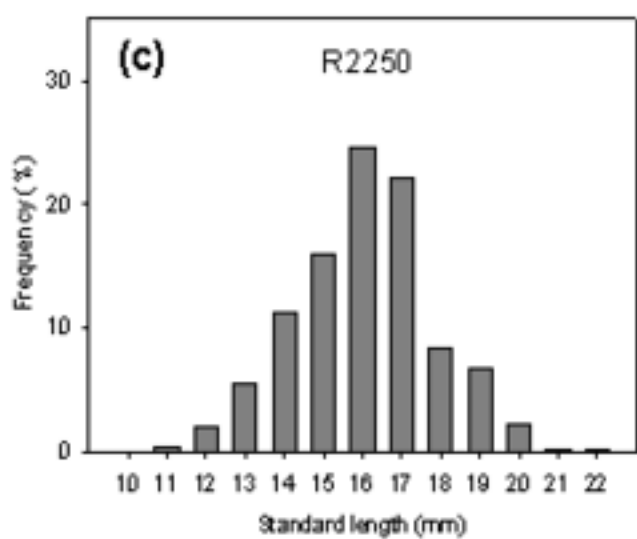
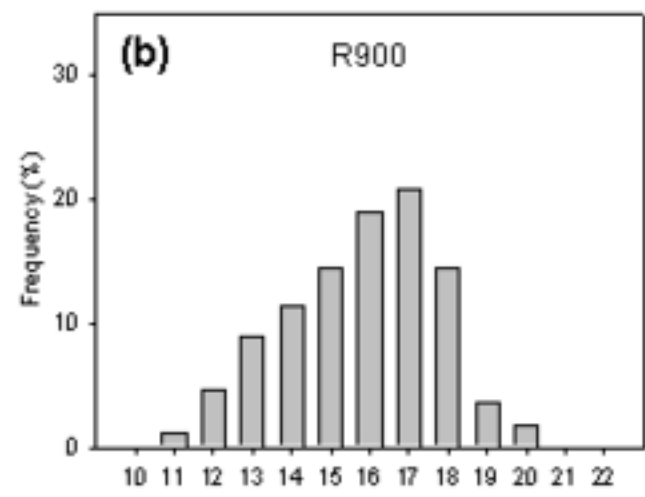
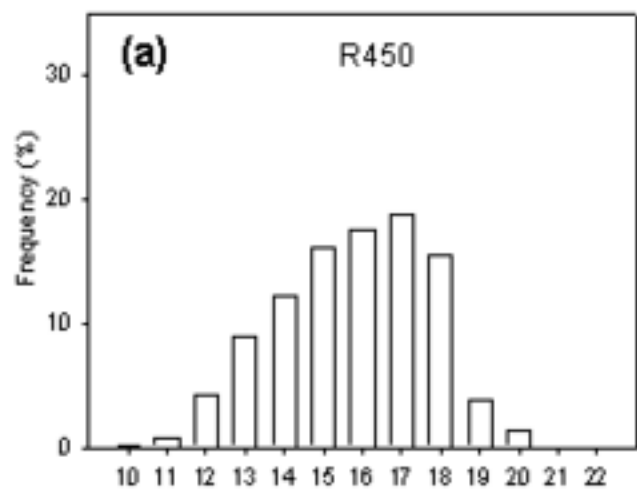


Figure 2
[Click here to download high resolution image](#)

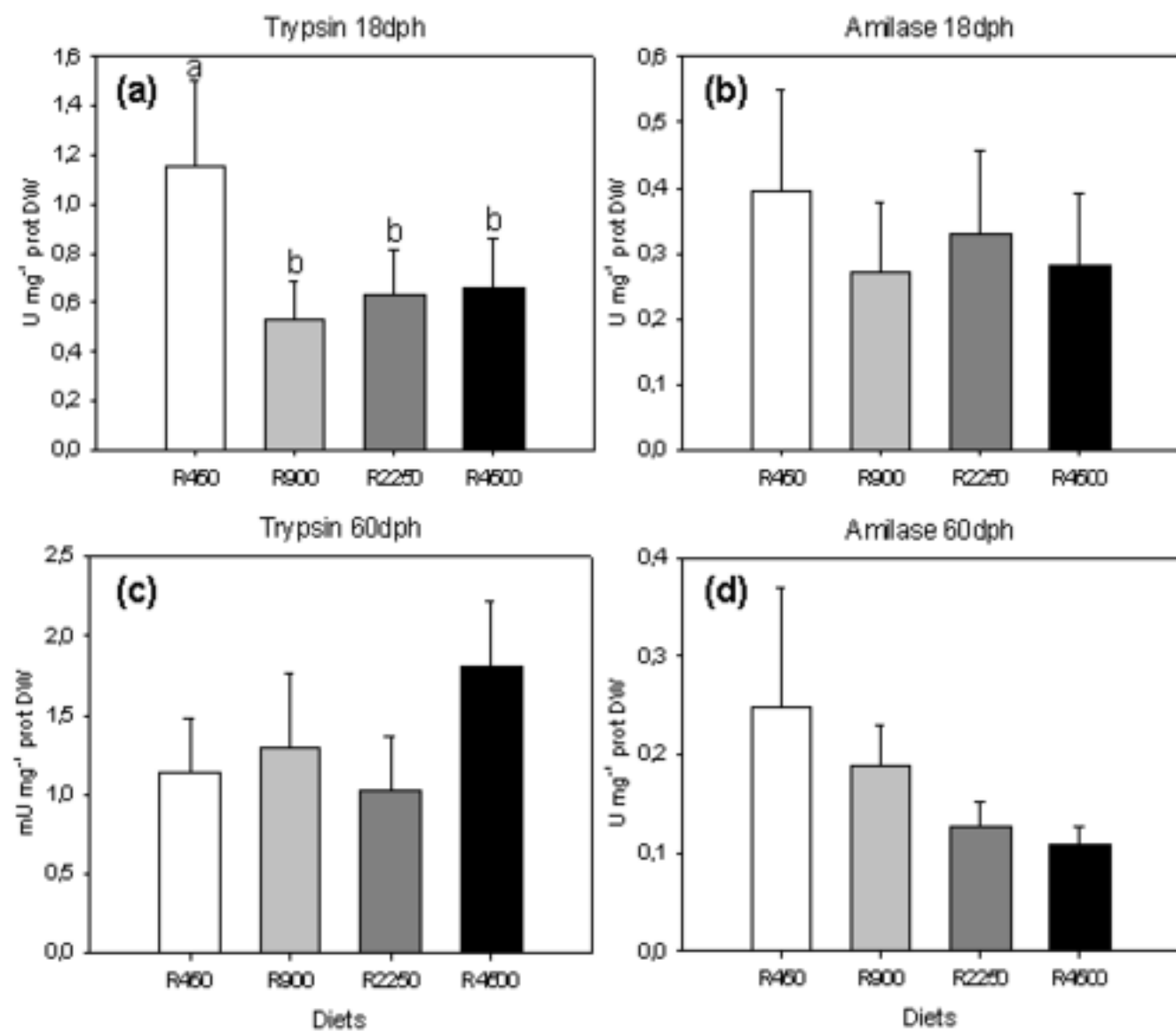


Figure 3
[Click here to download high resolution image](#)

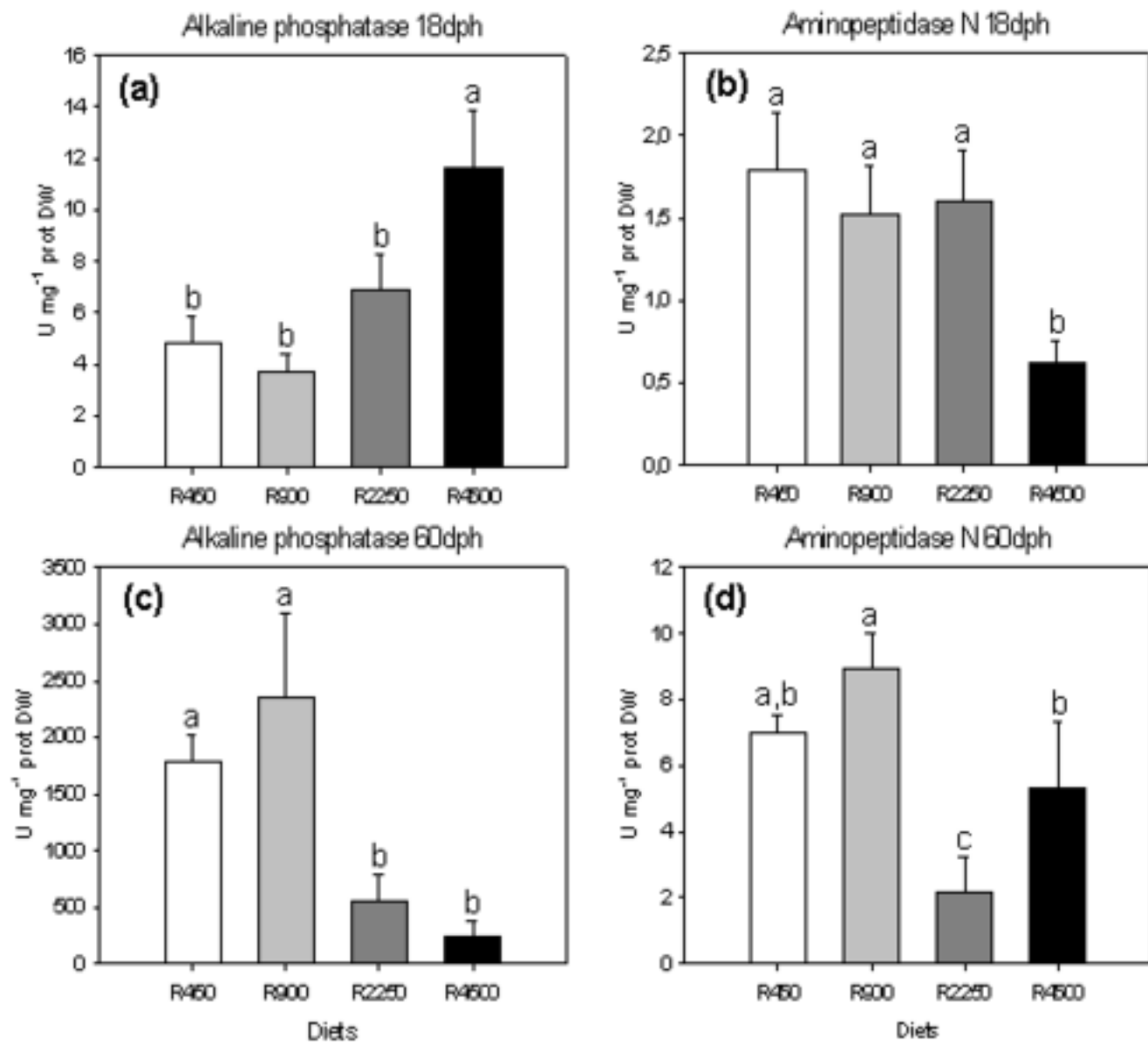


Figure 4
[Click here to download high resolution image](#)

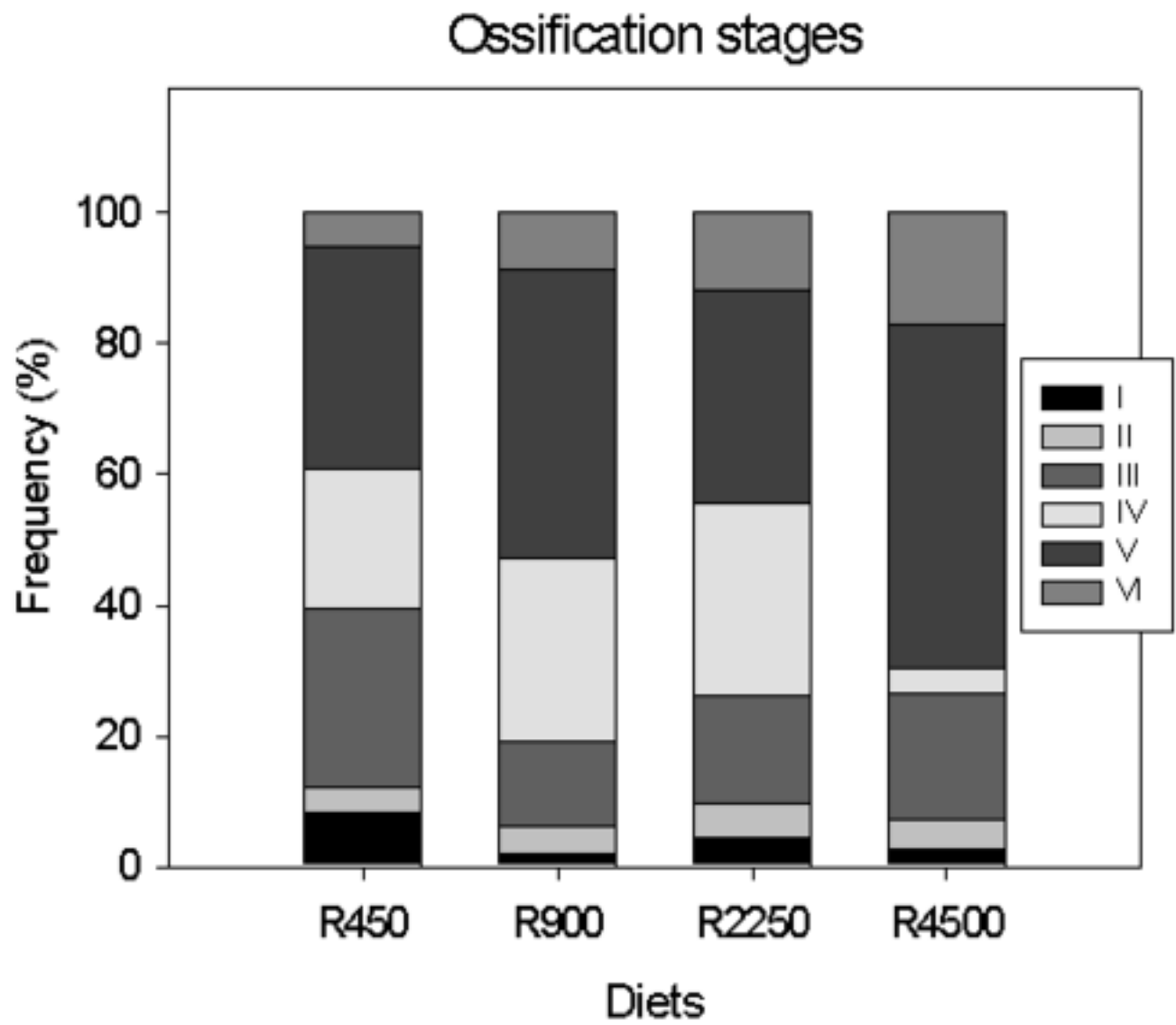


Figure 5
[Click here to download high resolution image](#)

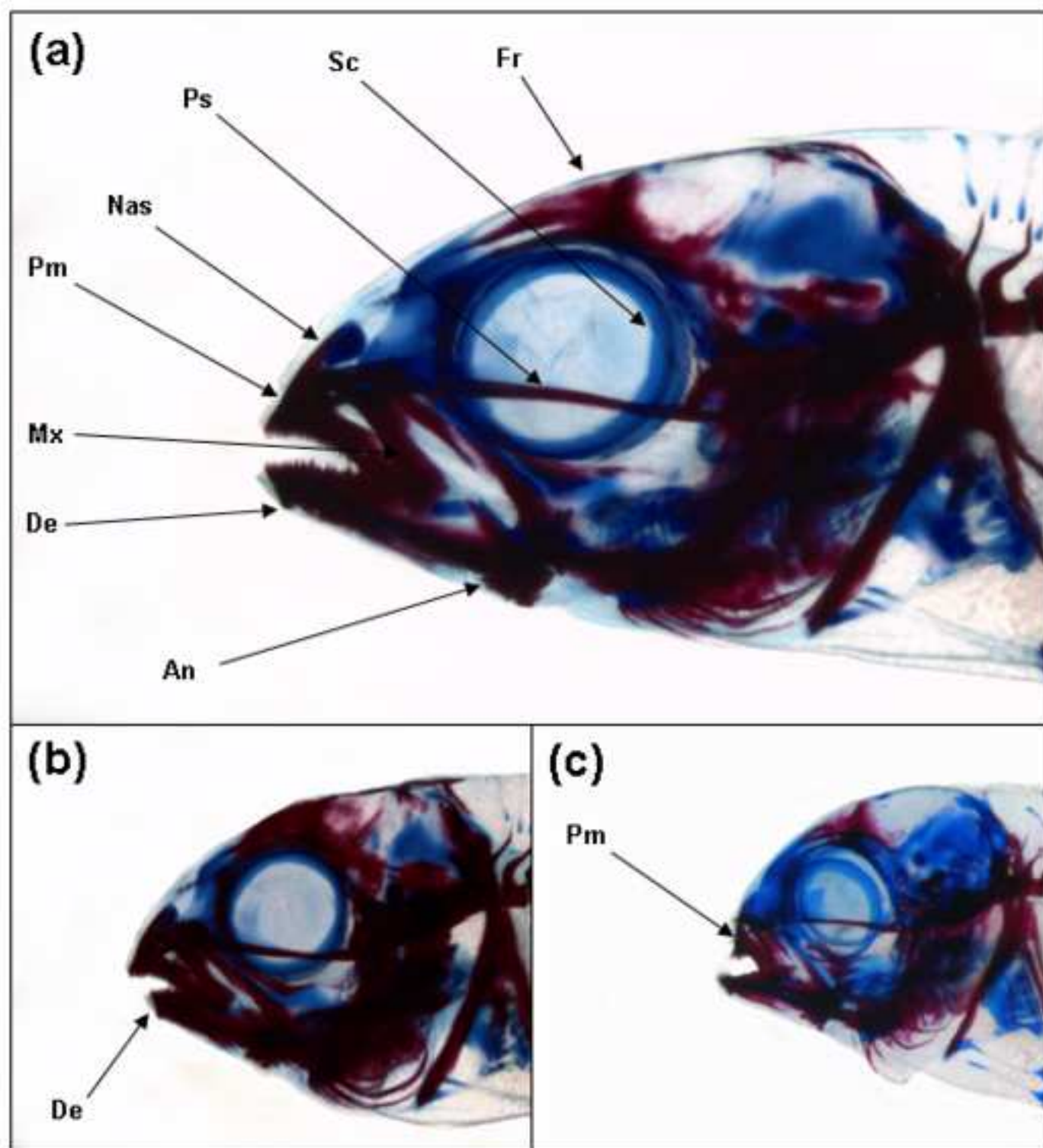


Figure 6

[Click here to download high resolution image](#)

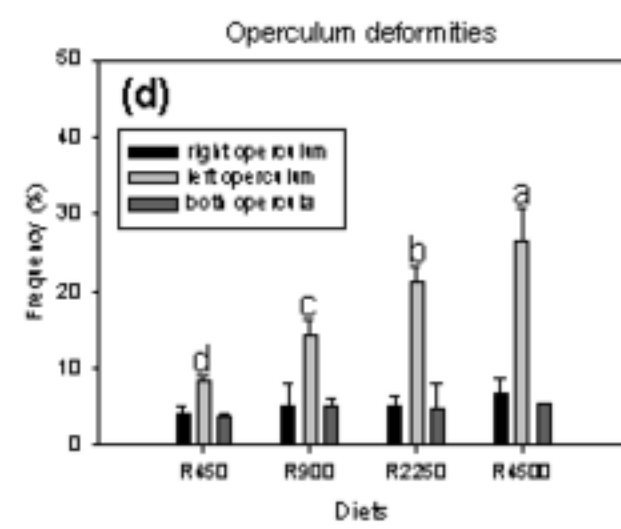
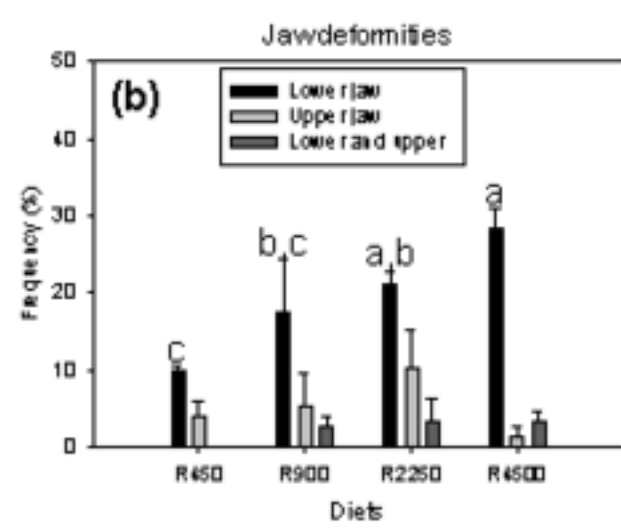
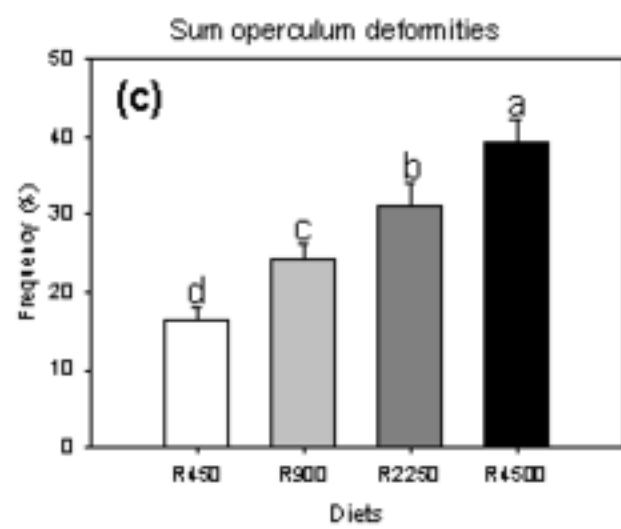
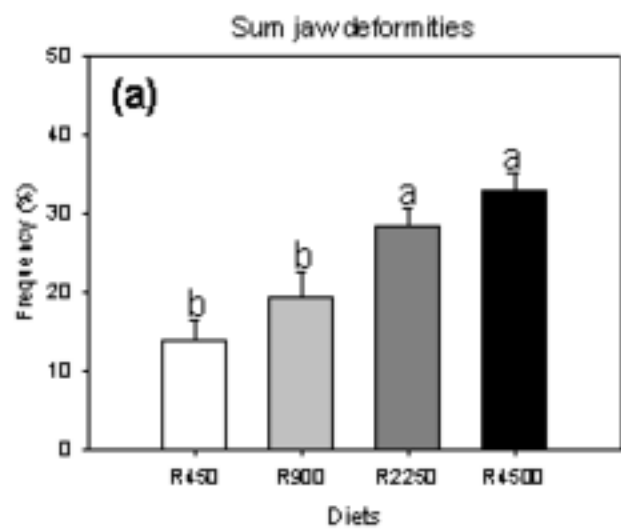


Figure 7

[Click here to download high resolution image](#)

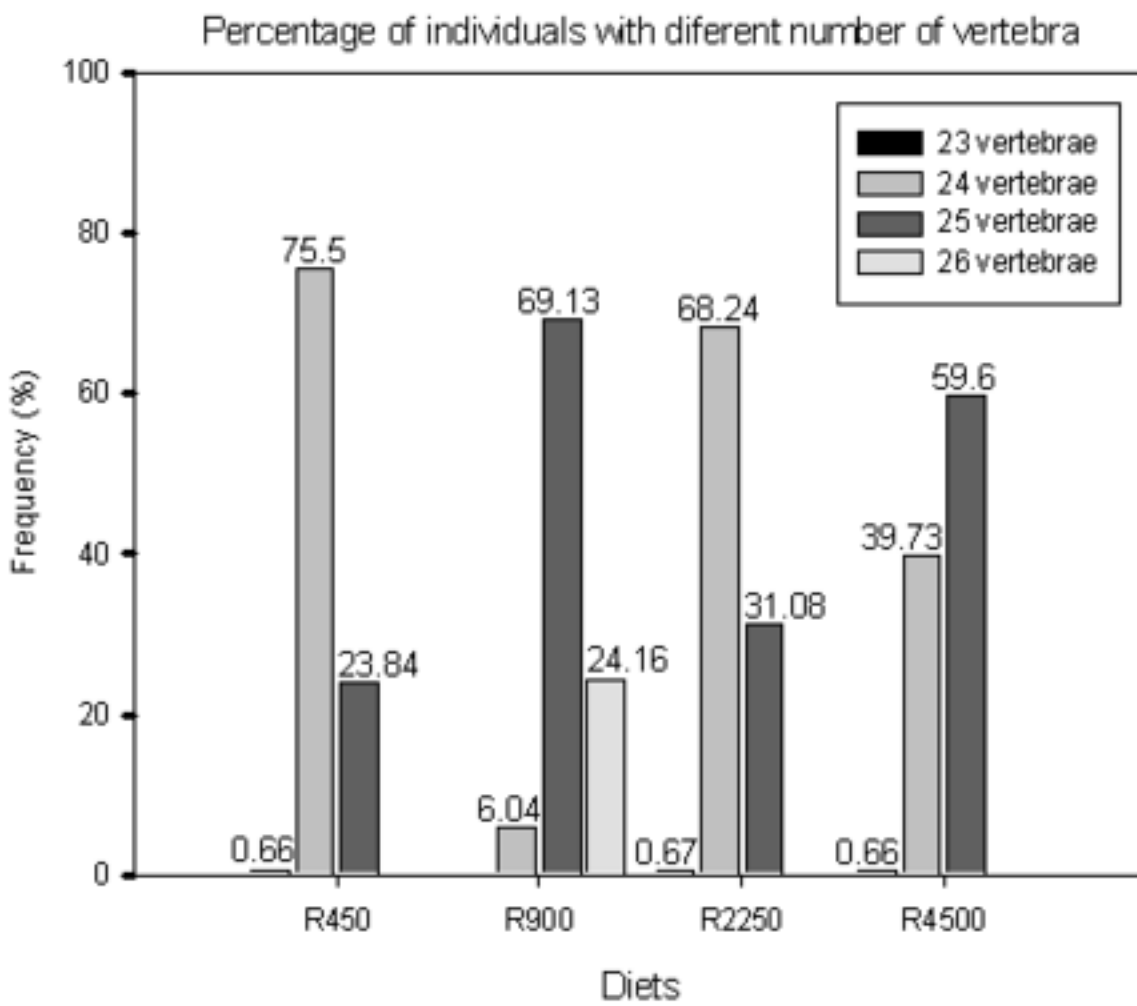


Figure 8

[Click here to download high resolution image](#)

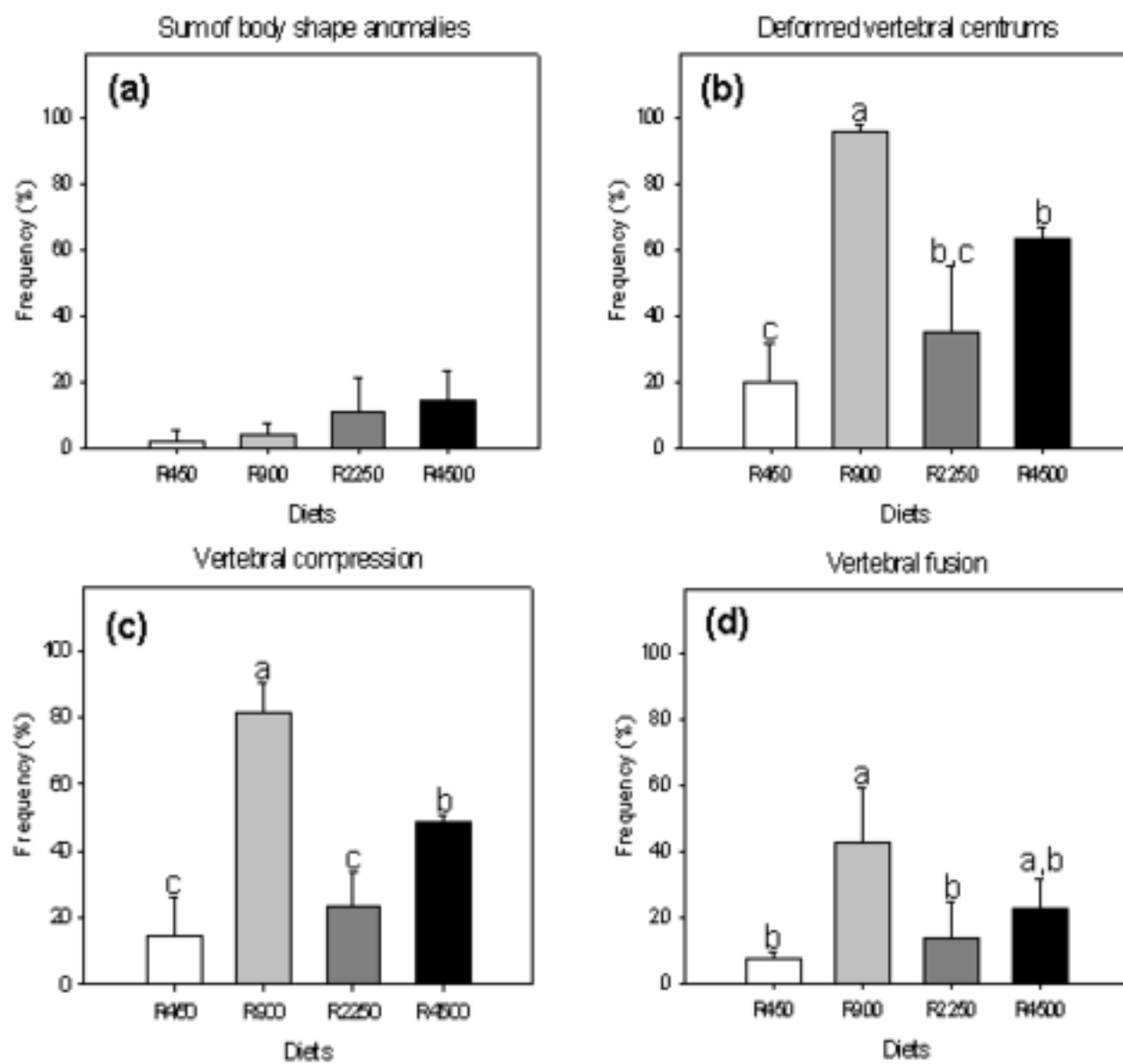


Figure 9

[Click here to download high resolution image](#)

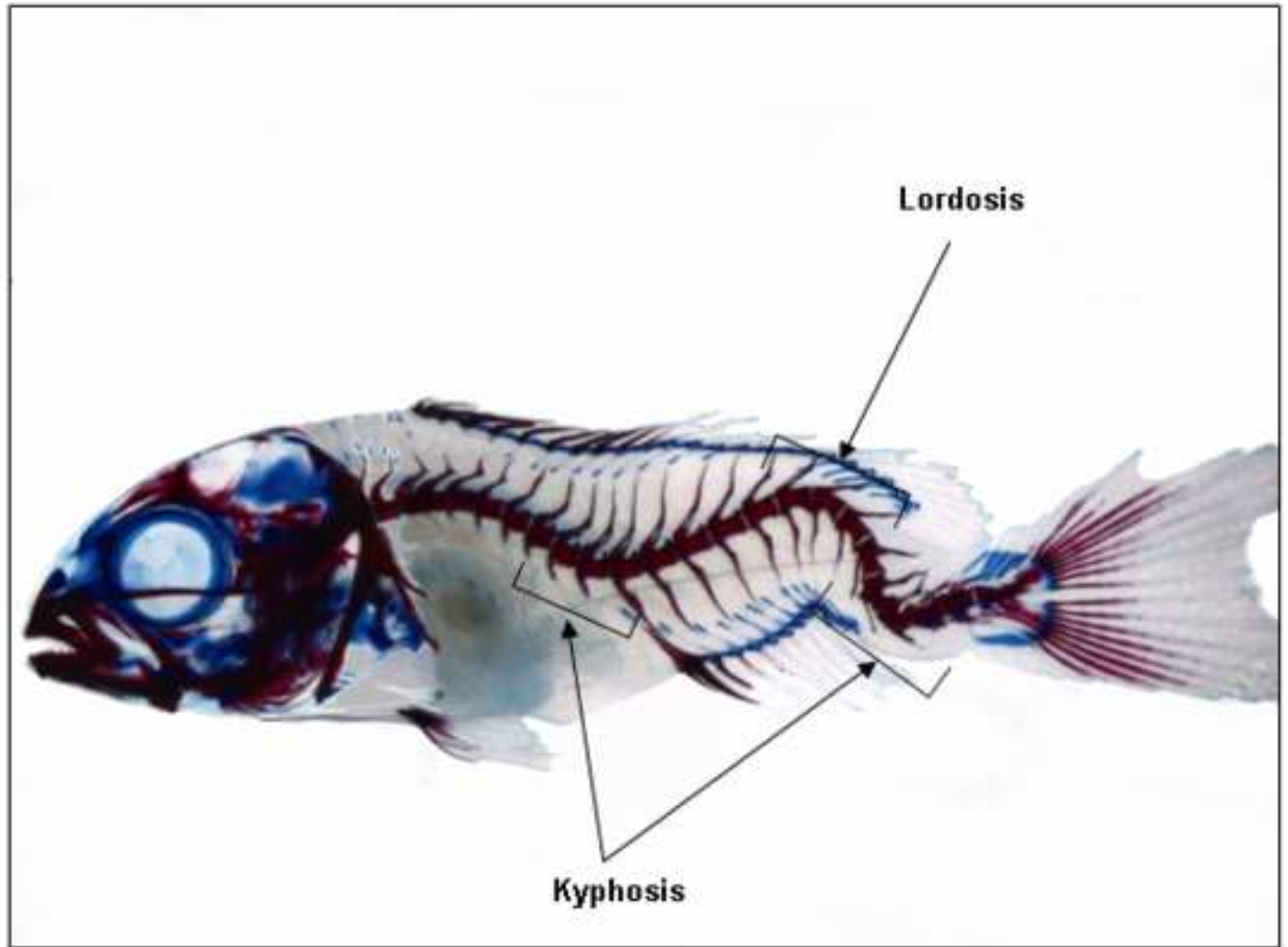
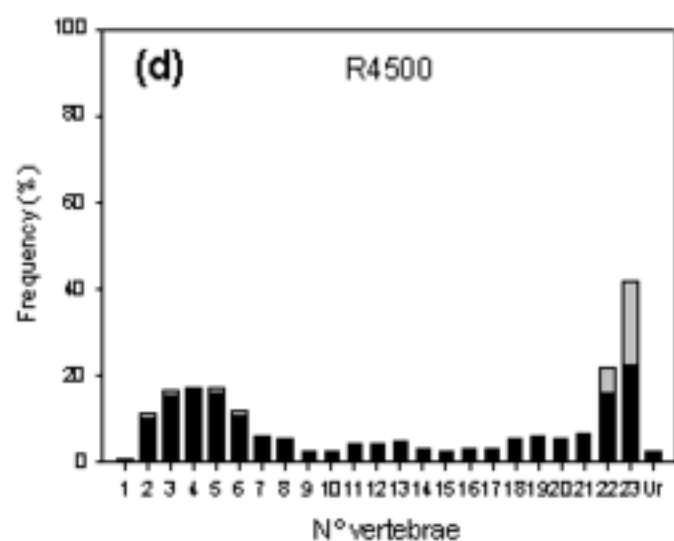
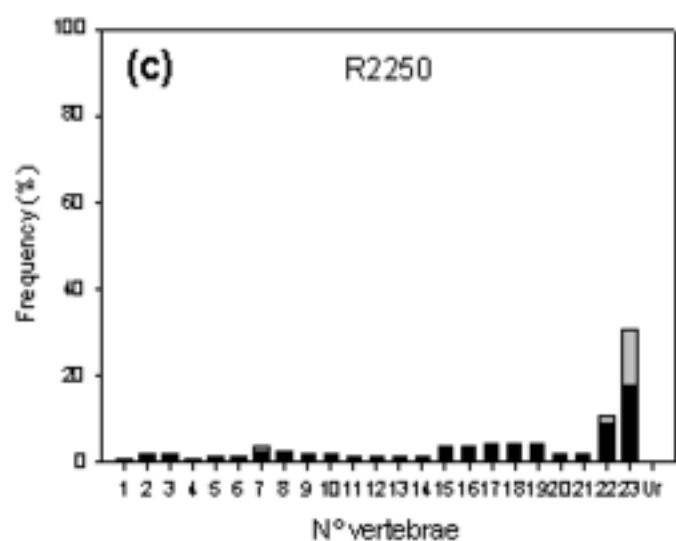
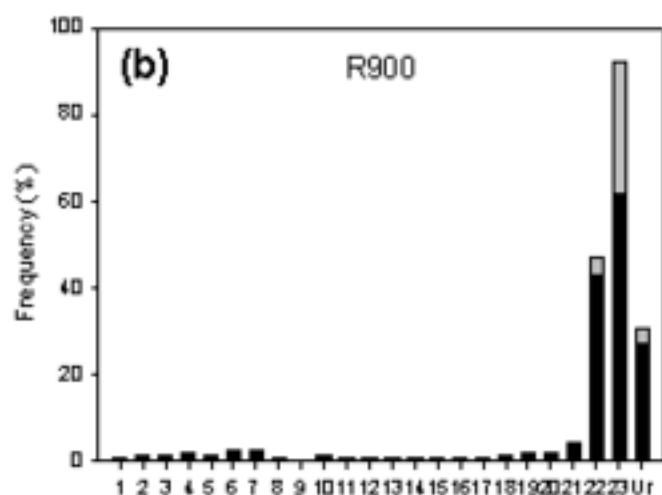
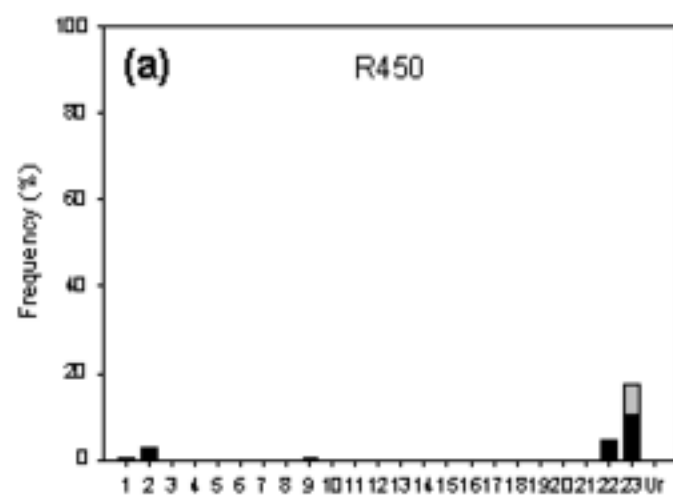


Figure 10

[Click here to download high resolution image](#)



ceph pre-haem haem caud

ceph pre-haem haem caud

Figure 11

[Click here to download high resolution image](#)

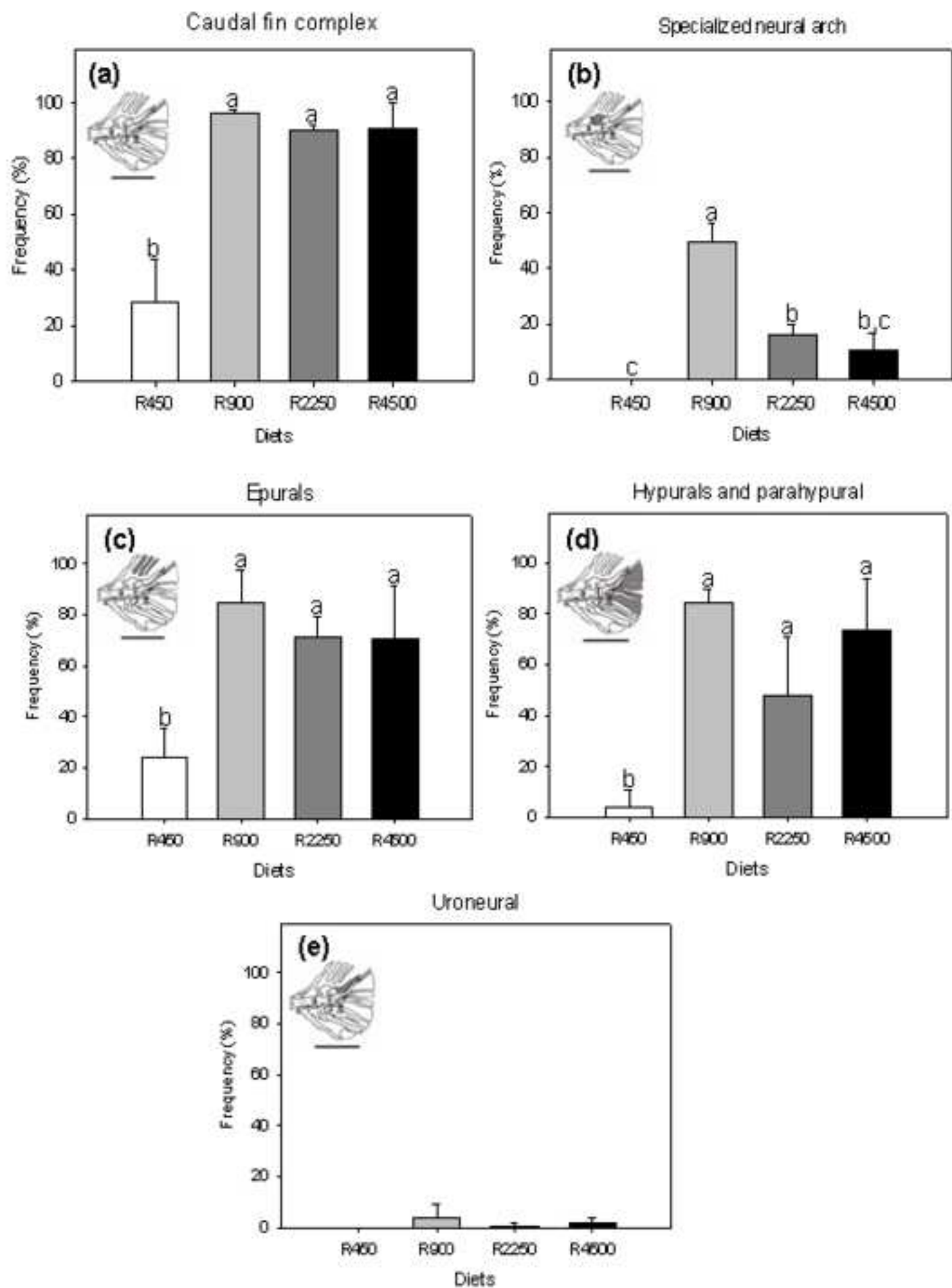


Figure 12
[Click here to download high resolution image](#)

